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"*Chlamydia pneumoniae* and *Simkania negevensis* in severe respiratory infection"

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B. Table of Contents

C. Executive summary

The overall aim of this research project was to determine the prevalence of infection with *Chlamydia(Chlamydophila) pneumoniae* and *Simkania negevensis* in certain population groups in Lima, Peru, and in the Negev region of Israel; to determine the possible association of these organisms with severe respiratory illness; and to investigate the possibility that drinking water (or waste water) may be a source of infection. In order to achieve these objectives, several new techniques were developed or adapted that served for serologic detection of antibodies (dot blot, MIF, ELISA, and IPA) or for the detection of microorganisms -- by the detection of their DNA (PCR) or their antigens (MEIA). The new techniques developed were shared with our colleagues in Peru and were then employed in their laboratory. With these techniques, much new data was obtained; our results were presented at international meetings, and some have now been published. Although we had previous data on the association of *S. negevensis* infection with bronchiolitis in infants and pneumonia in adults, this was extended to acute exacerbations of chronic obstructive pulmonary disease in adults. We also have now obtained serologic evidence that many *S. negevensis* infections occur during the winter months in Israel. While there are some indications of involvement of *C. pneumoniae* in severe pediatric respiratory infections in Lima, there is not yet clear evidence for significant involvement of *S. negevensis* in such infections. Further studies need to be carried out with the techniques developed during this project. We have now shown that *S. negevensis* can grow in free-living amoebae and survive for long periods in amoebal cysts (in addition to growth in cell lines, which we showed previously). In this study, we found evidence for the existence of Simkania or Simkania-like microorganisms in drinking water and in reclaimed waste water both in southern Israel and in Lima, Peru. The common presence of *S. negevensis* in water sources together with the high seroprevalence of antibodies to it and early age of acquisition of infection may implicate water as a source of infection. The possible significance of this finding for public health and for municipal water testing and treatment needs to be further examined.

D. Research Objectives

Respiratory tract infections are a serious problem in Lima, Peru, as in other developing areas of the world. In both developing and developed areas, a significant proportion of serious respiratory tract infections cannot be attributed to known etiologic agents. If the actual agents are viral, with no known specific treatment, there is no practical significance to the situation. However, if some of the agents are bacteria which are sensitive to common and inexpensive antibiotics, then empirical treatment should cover these agents as well, with significant advantages to the health and well-being of the population. Two such bacteria are *S. negevensis* and *C. pneumoniae*. The extent of infections with the first is only beginning to be appreciated, and little is known of the extent of involvement of the second in serious respiratory tract infections in the developing world. One aim of this study was to determine the prevalence of infection with these two agents in young children in Lima, Peru, and the extent of seropositivity to *S. negevensis* in several population samples. We had previously shown that infection with *C. pneumoniae* is common in Lima, but nothing was known of the prevalence of exposure *to S. negevensis* in Lima. An additional aim of the study was to develop simple serologic and simple diagnostic tests for the presence of antibodies to *S. negevensis* or for the presence of the organism in clinical samples. The final aim of the study was to develop simple tests and sensitive tests for detection of the organism in various water sources in Israel and in Lima.

It has recently been shown that many chlmydia-like microorganisms can be found in the environment ["environmental chlamydiae" (1)] mostly by PCR amplification of DNA sequences, but only in a few cases has a connection to human illness been made. This field of research is rapidly expanding today, because of the Legionella paradigm of enhancement of virulence for man by prior growth in free-living amoebae on the one hand, and on the other, because it has so far not been possible to assign an etiology for a significant proportion of serious human respiratory tract infections. The development of simple techniques for detection of such organisms in environmental or clinical samples should significantly advance such studies.

Ben Gurion University of the Negev supported this study by contributing the salaries of the principle investigator and Dr. Simona Kahane. The southern district office of the Israel Ministry of Health was very helpful in obtaining water samples and serum samples of pregnant women in the Negev, helping up to carry out the chlorination studies, and furnishing the evaluation data on water quality for the samples we tested.

E. Methods and Results:

1. Clinical and environmental samples

1.1. Peru

1.1.1. Serum samples:

1.1.1.1. Sera of 89 children with severe respiratory infection hospitalized in Lima were obtained during the course of a previous AID research project. From 68 of these children, paired sera were available for testing.

1.1.1.2. Sixty-five serum samples were collected for this study from young Peruvian children (up to 5 years old) diagnosed with severe acute respiratory disease, at El Nino Hospital and 77 such samples from children hospitalized at Dos de Mayo Hospital.

1.1.1.3. Sera of 84 presumably healthy persons aged 1-55 years, living in the shanty-town area of San Juan de Miraflores were collected during the course of a previous AID research project for detection of antibodies against *C. trachomatis* and *C. pneumoniae.*

1.1.2. Nasopharyngeal washes (NPW):

1.1.2.1. NPW samples were collected from children with severe respiratory tract infection in two hospitals in Lima, for PCR testing for the presence of *S. negevensis* DNA and *C. pneumoniae* DNA; 77 samples from Dos de Mayo Hospital and 63 samples from El Nino had enough material for testing. Children who met the SATWHO criteria for pneumonia or ALRI (2,3), and whose parents consented, were included in the study (with certain exclusions).

1.1.3. Water samples:

1.1.3.1. Initially, 79 samples of drinking water as well as 17 samples of untreated sewage water were collected from different parts of Lima. The drinking water included 15 samples of well water that were stored for various periods of time, ranging from 1 day to 2 months, 28 samples of cistern water, and 13 samples of tap water. The remaining samples of drinking water had been stored in buckets, in pots and in plastic containers, but the source was not specified. Later, a further 80 samples of drinking water and 20 samples of waste water were collected for testing for the presence of *S. negevensis.*

1.2. Israel

1.2.1. Serum samples

1.2.1.0. All of the following sets of serum samples (except 1.2.1.6.) were available for testing from previous serologic studies, and permission was obtained from our Institutional Review Board (IRB) for their use in the present study without additional informed consent.

1.2.1.1. Four to five serial serum samples were available from each of 92 healthy teenagers, aged 11-17 years, residents of five kibbutzim (agricultural communal settlements) in the northwestern Negev. The sera were originally obtained in a follow-up study of hepatitis vaccination, carried out from January, 1995, to January, 1996.

1.2.1.2. Paired serum samples from 240 patients with acute exacerbation of chronic obstructive pulmonary disease (COPD), and from 100 control patients hospitalized in an orthopedic ward at the Soroka University Medical Center.

1.2.1.3. Paired serum samples from 121 patients with upper respiratory disease (URI) who sought medical attention at primary care clinics in Beer Sheva. URI was defined as fever $\geq$37.5 °C and at least one of the following: cough, rhinitis, pharyngitis or hoarseness.

1.2.1.4. Paired serum samples from 100 patients seen at the emergency room (ER) of the Soroka Medical Center for respiratory tract infeciton, but not hospitalized.

1.2.1.5. Paired serum samples from 97 adults with community acquired pneumonia (CAP).

1.2.1.6. Single serum samples from 212 pregnant women living in various localities in the Negev. These sera, which are normally discarded after routine serologic tests, were saved for us by the laboratory of the Health Ministry (in accordance with permission from the local IRB).

1.2.2. Water samples

1.2.2.1. Thirty-five samples of drinking water were obtained from specific sources in Beer Sheva and surrounding satellite communities, and 40 samples of reclaimed waste water were obtained from various locations in the Negev (southern Israel), with the cooperation of the Southern District Office and Laboratories of the Israel Ministry of Health. Many more samples were tested during the course of the development of the MEIA. The samples mentioned above were those tested using the final protocol.

2. Methods developed / optimized / used

2.1. Serologic assays for antibodies to *S. negevensis* and/or *C. pneumoniae*

2.1.1. Dot-blot for detection of antibodies (developed).

This assay system was developed in the framework of this research project. The assay is suitable for use in remote areas and requires only an antigen-impregnated membrane, sample tubes, a pipettor for microliter volumes, and developing reagents. Results are read with the unaided eye. Antigen-impregnated and blocked membranes can be stored at room temperature for many months. Details on the optimization of this system can be found in the "Results" section, below.

2.1.2. Microimmunofluorescence (MIF) (used for C. pneumoniae, developed for S. negenesis)

The MIF test used for *C. pneumoniae* was based on commercially available slides (IO International, London), and goat anti-human FITC-conjugated antibodies were used for detection of human antibodies bound to the antigen on the slides.

The final protocol of the test developed for detection of antibodies was as follows: Glass slides with very small wells were thoroughly washed (twice with distilled water, once with water:acetone at 1:1, and once with pure acetone. Elementary bodies of *S. negevensis*, purified by standard techniques (4), were fixed by incubation with 1% formalin and incubation for 2 hr at room temperature. The suspension was diluted and filtered in order to obtain a uniform suspension of individual particles. These were applied to wells on the slides at a uniform protein concentration, dried slowly, and fixed with cold acetone. Serum dilutions were applied to individual wells, incubated for one hour and washed. Second antibody was goat anti-human IgG conjugated to FITC (Jackson Labs) in 0.1% Evans blue. After another 1 hour incubation, washing and drying, mounting medium was added, and slides were observed under the microscope for typical "starry sky" positivity. Control positive and negative serum pools (as determined by ELISA) served as positive and negative controls in this assay. Results were read independently by at least two laboratory workers.

2.1.3. ELISA

The ELISA used for detection of antibodies to *C. pneumoniae* and *C. trachomatis*, and to *S. negevensis* has been described previously (5-7). It has been shown to be specific for each organism, depending on the antigen used to coat the ELISA plates. However, because it includes a urea rinse step after incubation of the serum being tested (for increased specificity), it is best suited to detection of antibodies of relatively high affinity. It can be used for the detection of antigen-specific IgG or IgA, but not IgM. In this project, we found that the IgA cutoff level for sera of infants and young children is lower than for adults.

2.1.4. Immunoperoxidase assay (IPA) for IgA (used).

This assay has been described previously (8). Antigen in this assay consists of a mixture of infected and uninfected cells, applied to glass slides in drops, dried and fixed. Serum dilutions are applied to the dried antigen. After incubation and washing, the slides are treated with second antibody conjugated to horseradish peroxidase, incubated, and washed. Detection of positive reactions is with a diaminobenzidine substrate, with microscopic observation of staining results. This assay is accurate for serum samples of subjects of any age.

2.2. Growth and detection of *S. negevensis* in *Acanthamoeba polyphaga* (developed).

A detailed description of the methods used to grow *S. negevensis* in *A. polyphaga* and methods for detection of the organism in the amoebae can be found in the article attached (9). The methods developed allow quantitation of the number of amoebae present, calibration of the amounts of antigen present, and detection of DNA present.

2.3. MEIA for antigen detection (developed).

The membrane enzyme immunoassay for detection of *S. negevensis* in water samples is fully described in the article attached (10). This is an original method developed for this study. The final protocol developed was found to be specific and sensitive for the detection of *S. negevensis* not only in water samples; it was also found suitable for detecting antigens in NPW of children (unpublished results; this is a separate study in progress.)

2.4. PCR for sensitive DNA detection (newly developed nested PCR).

An original procedure was developed for preparation of DNA from water samples. This DNA was tested in new, more sensitive PCR assays, which were developed for this study and are fully described in reference 10, attached). The original PCR assay used for the detection of *S. negevensis* in water samples and in clinical samples in Peru in this project was described previously (8).

DNA from drinking water samples was prepared as follows: a volume of 500 ml water sample was drained (by gravity) through a cellulose acetate filter (cat. # 12342-47-N, Sartorius, 5 micrometer); the filter was incubated with Page's saline buffer (24) for 1 hr; the solution containing the biological material was centrifuged to sediment amoebae; and the precipitate was used to prepare DNA with the QIAamp kit (Qiagen).

Assays for the detection of the *S. negevensis* genome by PCR were performed using two sets of nested primers: The first set consisted of primers ccF and ccR recognizing conserved chlamydial 16S rDNA sequences (433-457 and 933-945, respectively) and amplifying a 512 bp fragment, followed by nested ZpF and ZpR primers specific for *Simkania negevensis* Z [as previously described (8)], amplifying a 405 bp fragment. A second set of nested primers consisted of AF and BR amplifying a 1099 bp fragment within the 23S rDNA of *S. negevensis* containing the LSU intron (11) and IntD and IntR nested primers amplifying a 338 bp fragment within this intron. If the DNA tested does not contain the intron, a 441 bp fragment is obtained with the AF and BR primers, and no sequence is amplified by the IntF/IntR primer pair.

The use of the nested primers allowed increased sensitivity of the PCR assay when it was necessary; however, when DNA was abundant, primer sets AF/BR or IntF/IntR or ZpF/ZpR could also be used alone. PCR assays for the presence of amoebal sequences were carried out with the primers AmP1 and Amp2 described by Lai *et al.* (12) using the cycling program described for *S. negevensis* (10). When amplification was with the ccF/ccR primers, the same amplification program was used except that annealing was performed at 58 °C. instead of 53 °C. When nested primers were used, the material amplified with the first pair of primers was diluted 1:100, of which 1 microliter was used in the second reaction mixture. The high dilution was needed in order to ensure the specificity of the assay.

3. Results

3.1 Results obtained with various serologic techniques

3.1.1. Seasonality of infection with C. pneumoniae and S. negevensis in the northwestern Negev.

From the examination of four to five serial serum samples from each of 92 healthy teenagers for IgG and IgA antibodies to *Chlamydia pneumoniae* and to *Simkania negevensis* by the specific ELISA assays which we developed, an interesting picture of the seasonality of infection has emerged. A rise or fall in IgG level was arbitrarily defined as 0.3 adjusted OD units. Tables 1 and 2 show that most rises in IgG antibody levels occurred between January and February. Although the number of subjects in each kibbutz was relatively small, it is notable that winter rises in antibody level to *C. pneumoniae* were limited to one kibbutz, while winter rises in antibody level to *S. negevensis* were evenly distributed among the kibbutzim. Only in one person were significant changes in antibody levels to both organisms observed, but not in the same time interval.

Table 1. Evidence for intercurrent infection with *C. pneumoniae* by season, in 92 teenagers ranging in age from 11-17 years, living in five kibbutzim in the Negev, expressed as the number of subjects with rising (r) or falling (f) levels of specific IgG serum antibody to the organism.

Kibbutz	n	Jan-Feb	Feb-Apr	Apr-Jul/Aug	Jul/Aug-Jan
1	24	6 r	1 f	---	---
2	17	---	---	---	1 f
3	17	---	---	---	1 f
4	18	1 f	---	---	---
5	16	---	1 r	1 f	---
Total	92	6 r 1 f	1 r 1 f	1 f	2 f

Table 2. Evidence for intercurrent infection with *S. negevensis* by season in 92 teenagers ranging in age from 11-17 years, living in five kibbutzim in the Negev, expressed as the number of subjects with rising (r) or falling (f) levels of specific IgG serum antibody to the organism.

Kibbutz	n	Jan-Feb	Feb-Apr	Apr-Jul/Aug	Jul/Aug-Jan
1	24	2 r	1 f	1 r	2 f
2	17	2 r	---	2 f	---
3	17	2 r	1 f	---	---
4	18	2 r	---	1 r	---
5	16	2 r	1 f	---	1 r
Total	92	10 r	3 f	2 r 2 f	1 r 2 f

3.1.2. Number of seroconversions per year.

Table 3 shows the seropositivity rate to the two organisms in the various kibbutzim at the beginning and at the end of the follow-up period. Although in some kibbutzim several more children were positive at the end of the year than at the beginning, overall, there was a net drop (slight) in the number of youth seropositive according to the ELISA assay (with respect to both organisms) at the end, compared with the beginning. It may be that the cutoff levels which we used for determination of seropositivity were too high for this population group, or it may be that seropositivity to these organisms tends to dwindle with time. Further testing of these sera by additional serologic techniques may shed light on the issue. In any case our results seem to indicate that by the beginning of the teen years, many people are already seropositive to the organisms (although even in the same geographic region, there may be rather extreme differences -- viz. kibbutz number 5, compared to kibbutz number 1). In that connection, it is interesting that kibbutz number 5, of all the kibbutzim, is nearest to the Gaza strip and has seropositivity rates most similar to those of the Negev Bedouin (7, 13).

Table 3. Percent IgG seropositivity to *C. pneumoniae* and to *S. negevensis* in 92 teenagers in five Negev kibbutzim at the beginning (beg) and at the end of the follow-up period, as determined by ELISA assay.

		C. pneumoniae		*S. negevensis*	
Kibbutz	n	beg	end	beg	end
1	24	50	54	29	33
2	17	59	47	41	35
3	17	59	53	41	47
4	18	37	37	26	21
5	16	27	27	60	53
Total	92	47	45	38	37

3.1.3. Possible involvement of S. negevensis in acute exacerbation of chronic obstructive pulmonary disease (COPD).

In our comparison of 100 paired sera of patients with acute exacerbation of COPD and 100 paired sera of control patients hospitalized in an orthopedic ward, for detection of IgG and IgA antibodies to *S. negevensis* and to *C. pneumoniae* by ELISA, four COPD patients (=4%) had serologic evidence of infection with *S. negevensis* during their current exacerbation, while none of the control patients had such evidence of current infection. Serologic evidence was a change of $\geq$0.5 adjusted OD units in the ELISA between paired serum samples. One patient had serologic evidence for *C. pneumoniae* infection during his current exacerbation, while none of the control patients did. This constitutes evidence for possible involvement of *S. negevensis* in some cases of exacerbation of COPD. Possible involvement of Cpn in such exacerbations has been previously described. (14) All of the patients with serologic evidence for association of *S. negevensis* infection with their acute exacerbation of COPD were admitted to the hospital between mid- and late January. Paired serum samples of a further 140 patients were tested by ELISA for IgG and IgA antibodies to both organisms. A further three patients were found with serologic evidence of acute *S. negevensis* infection; one was admitted to the hospital in late August, and two others in late January and mid-February of the following year. More details can be found in the article attached (15).

3.1.4. Possible involvement of S. negevensis in upper respiratory illness in adults

Paired sera of 121 patients with upper respiratory illness (URI) who sought medical attention in primary care clinics in Beer Sheva were examined by ELISA. URI was defined as fever $\geq$37.5°C and at least one of the following: cough, rhinitis, pharyngitis or hoarseness. IgG antibodies to *S. negevensis* were found in 88 of the acute sera (73%), and IgA antibodies were found none of the acute sera. Possible serologic evidence for involvement of *S. negevensis* was found in one patient who developed IgA antibodies to the organism in his convalescent serum sample. Thus there was very little serologic evidence for involvement of *S. negevensis* in the upper respiratory tract infection of these patients.

Paired serum samples of 100 patients seen at the emergency room (ER) of the Soroka Medical Center for respiratory trat infection, but not hospitalized, were tested by the ELISA for IgG and IgA antibodies to *C. pneumoniae* and *S. negevensis* . Seventy-one percent were seropositive to *S. negevensis* (IgG) and 20% had IgA antibodies to the organism in their first serum sample. In comparison, 70% were seropositive to *C. pneumoniae*, and 17% had IgA antibodies to this organism in their first serum sample. Two patients had rises of IgG levels greater than 0.3 OD to *S. negevensis* and one had such a rise of IgA antibodies. None had titer changes greater than 0.5 OD. Two different patients had differences of >0.3 OD in IgG levels to *C. pneumoniae* —one was a rise of 1.39 OD and one was a fall of 0.42 OD.

Taken together, these serologic results seem to indicate that *S. negevensis* may be involved in upper respiratory tract infection, as is *C. pneumoniae* to some extent, but that this may be best detected by methods other than serology. Furthermore, patients accessing the ER for medical attention may be more ill (have a more deep-seated infection) than patients approaching their local clinic for treatment.

3.1.5. Seropositivity to S. negevensis and C. pneumoniae in pregnant women in various towns in the Negev.

Sera from 137 women from seven different localities in the Negev were tested for seropositivity to *S. negevensis* and to *C. pneumoniae* by the ELISA assay. Table 4 shows that seropositivity to *S. negevensis* was in general higher than seropositivity to *C. pneumoniae*. The table furthermore shows the ethnicity of the residents of the towns. Only in Arad, is the population mixed, ethnically, and the breakdown for that town is shown in the table.

Table 4. Seropositivity to *C. pneumoniae* and to *S. negevensis* among pregnant women in seven towns in the Negev, as determined by ELISA.

Town	Ethnicity*	Number of sera	Percent positive *S. negevensis*	Percent positive *C. pneumoniae*
Tel Sheva	B	32	78	28
Rahat	B	27	63	30
Ksiffia	B	11	73	27
Netivot	J	14	50	21
Laqia	B	11	73	36
Hura	B	22	100	45
Arad	J	3	67	67
Arad	B	17	82	18

* B = Bedouin, J = Jewish

There is a definite tendency for very high seropositivity rates to *S. negevensis* in the Bedouin towns/settlements (between 63 and 100%), and lower rates to *C. pneumoniae* (varying between 18 and 45%), in agreement with past observations (7, 13).

3.1.6. Serology for residents of San Juan de Miraflores.
When sera of 84 presumably healthy residents of San Juan de Miraflores were tested for the presence of IgG antibodies to *S. negevensis* and to *C. pneumoniae* by the ELISA method developed previously (5, 7), the results shown in Table 5 (stratified by age group) were obtained.

Table 5. Seropositivity rates to *S. negevensis* and *C. pneumoniae* by age group for 84 healthy residents of San Juan de Miraflores in Lima, Peru.

Age groups		*S. negevensis*		*C. pneumoniae*	
(years)	n	No. positive	% positive	No. positive	% positive
1-4	18	6	33	9	50
5-10	20	10	50	10	53
11-20	16	6	37.5	11	69
21-29	19	10	53	12	63
30-55	11	5	45	11	100
Total	84	37	44	53	63

As seen in Table 5, infection with *C.pneumoniae* is very widespread, and seems to occur at an earlier age than in Israel. In this admittedly rather small population sample, 50% of children between the ages of 1 and 4 were already seropositive to this organism, as were all of the adults aged 30-55 years who were tested. On the other hand, the pattern of seropositivity to *S. negevensis* was unusual, in that the rates did not appear to be incremental, but rather seemed to hover between 35 and 50% for all the age groups, including the youngest. We have no explanation for these results. The rates of exposure to *C. trachomatis* (not shown here) were incremental, although lower than those for *C. pneumoniae*.

3.1.7. Serology for paired sera of children hospitalized in Peru with respiratory tract infection.
When the ELISA was used to test paired sera of 68 children hospitalized with severe respiratory tract infection in Peru for antibodies to *S. negevensis* (sera obtained in the framework of our previous grant from AID), several interesting observations were made. Firstly, children with IgA titers of 10 or more by IPA (sera were tested in doubling dilutions starting at 1:10) had adjusted OD values in the ELISA of $\geq$0.080. There was only one exception in which a child with IgA antibody by the IPA test had an ELISA OD value of 0.063. No children had OD values as high as 0.500 (the normal lower cutoff for positive adult sera), and no children who were negative by the IPA had ELISA adjusted OD levels above 0.08. Furthermore, when the children were stratified by age, while there was a trend toward increasing IgA positivity with age, most infections apparently took place at the age of 3-4 years. Later there was a clear reduction in IgA positivity by IPA and ELISA assays (see Table A). When IPA titers were compared for acute vs. convalescent sera for these children, most children had identical IgA titers in their paired serum samples; however, 5 children became IgA negative between the two serum samples (from a titer of 10) and one went down from 40 to 20; three went up from negative to 10, and one from 10 to 20. None of these differences reached laboratory significance, which is a four-fold change of titer. All of these sera were tested for IgA to *S. negevensis* by the dot blot assay which we developed as well. For the dot blot assay, a positive result was considered a titer of 256 and up. The results, shown in Tables 6 and 7, suggested that the antigen of the dot blot assay (whole bacterial cell lysates) bound antibodies produced in response to infections with organisms other than *S. negevensis*.

Table 6. IgA seropositivity to *S. negevensis* in an acute serum sample for 66* children with severe RTI in Lima, Peru, as tested by ELISA, IPA and dot blot assays.

Age group (yr)	n	ELISA OD $\geq$0.080	IPA $\geq$10	Dot blot $\geq$256
Up to 1	14	2 (14 %)	2 (14 %)	3 (21 %)
1-2	25	8 (32 %)	8 (32 %)	13 (52 %)
3-4	14	11 (79 %)	12 (86 %)	12 (86 %)
5-10	13	5 (38 %)	5 (38%)	10 (77 %)
Total	66	26 (39 %)	27 (41 %)	38 (58 %)

*For two patients data on age was unavailable.

Table 7. IgA seropositivity to *S. negevensis* in a convalescent serum sample for 66* children with severe RTI in Lima, Peru, as tested by ELISA, IPA and dot blot assays.

Age group (yr)	n	ELISA OD $\geq$0.080	IPA $\geq$10	Dot blot $\geq$256
Up to 1	14	0 (0 %)	2 (14 %)	3 (21 %)
1-2	25	3 (12 %)	6 (24 %)	15 (60 %)
3-4	14	7 (50 %)	9 (64 %)	10 or11 (71/79)
5-10	13	6 (46 %)	6 (46%)	11 (85 %)
Total	66	16 (24 %)	23 (35 %)	39/40 (59/61)

*For two patients data on age was unavailable.

Tables 8 and 9 show data on IgA antibodies to *C. pneumoniae* in these children, when stratified by age as in Table A. It would appear that in this population of patients, infection with *C. pneumoniae* is quite common already at 1-2 years of age, but is even more common in older children. However, we cannot assume that the OD 0.080 ELISA cutoff determined for *S. negevensis* IgA is appropriate for *C. pneumoniae* IgA as well. Therefore in Tables 8 and 9 we present data on the basis of a 0.400 OD cutoff as well. Even with this cutoff, 62% of the children aged 5-10 hospitalized for severe RTI had IgA antibodies to *C. pneumoniae*. However, in the second serum sample (Table 9) only 21% of the children still had IgA antibodies at the level of 0.400 OD.

Table 8. IgA seropositivity to *C. pneumoniae* in an acute serum sample for 66* children with severe RTI in Lima, Peru, as tested by ELISA assay, and using a cutoff of O.D. 0.080 vs. a cutoff of O.D. 0.400.

Age group (yr)	n	ELISA OD $\geq$0.080	ELISA OD $\geq$0.400
Up to 1	14	0 (0 %)	0 (0 %)
1-2	25	12 (48 %)	2 (8 %)
3-4	14	7 (50 %)	5 (36 %)
5-10	13	11 (85 %)	8 (62 %)
Total	66	30 (45 %)	15 (23 %)

*For two patients data on age was unavailable.

Table 9. IgA seropositivity to *C. pneumoniae* in a convalescent serum sample for 66* children with severe RTI in Lima, Peru, as tested by ELISA assay, and using a cutoff of O.D. 0.080 vs. a cutoff of O.D. 0.400.

Age group (yr)	n	ELISA OD ≥0.080	ELISA OD ≥0.400
Up to 1	14	4 (29 %)	0 (0 %)
1-2	25	15 (60 %)	1 (4 %)
3-4	14	12 (85 %)	6 (43 %)
5-10	13	13 (100 %)	3 (23 %)
Total	66	44 (67 %)	10 (15%)

*For two patients data on age was unavailable.

It is notable that IgA levels to *C. pneumoniae* were higher than to *S. negevensis*, especially among older children, with adjusted ODs of >1.000 being reached by some children already at the age of 2 years. Altogether, five children had *C. pneumoniae* IgA levels above 1.000 OD in their acute serum samples, but none had this level in a convalescent serum sample, for which O.D. 0.880 was the highest level observed. This data may indicate that *C. pneumoniae* was indeed involved in the current respiratory tract illness, especially if hospitalization was delayed relative to onset of illness, and if appropriate antibiotic treatment was administered in the hospital. Taken together, these results seem to indicate that *C. pneumoniae* infection may contribute to serious respiratory tract infections in young children in Lima, while there is less solid evidence that *S. negevensis* infections do so.

3.1.8. Evaluation of the microimmunofluorescence (MIF) assay developed as a potential "gold standard" for detection of antibodies to S. negevensis.

MIF has been considered the "gold standard" in chlamydial serology in spite of its being a relatively subjective and labor-intensive method. We developed a MIF technique for *S. negevensis* serology and compared its results with results obtained with ELISA and dot blot assays that we developed for detection of antibodies to the organism. Among the parameters optimized for this assay were: type of slides, form (EB, RB, or a mixture) and concentration of antigen , method of application of the antigen to the slide, fixation solution, commercial source and concentration of FITC-conjugated antibody, dilutions of serum to be tested (from 1:16), and long-term storage conditions for prepared slides.

The assay was evaluated using 97 sets of paired sera of patients with community acquired pneumonia (CAP) that have previously been described (6, 16). Six patients were found to have rising titers to *S. negevensis* (at least 4-fold) between acute and convalecsent serum specimens. The specificity of the MIF was tested by examination of a number of sera that were ELISA negative for antibodies to S. negevensis, but EISA positive for antibodies to *C. pneumoniae* or *C. trachomatis*. All had MIF titers ≤32 to *S. negevensis*, which was considered negative for IgG. Reproducibility of the MIF titers was tested with 16 positive sera and 4 negative sera. All 4 negative sera were again negative on repeat testing. For the positive sera, 15 of the 16 had the same titer on repeat testing or were within one 2-fold dilution, while one had a titer different by 4-fold on repeat testing.

The MIF assay is often considered to be a "gold standard" because one observes fluorescence of individual bacterial particles, and not general color development (as in ELISA) or staining of infected cells (as in IPA); however, it is laborious and time-consuming, and the reading of the results is somewhat subjective. It was not used further in this study, because it offered no real advantages over the ELISA and dot-blot assays, which proved to be much simpler to perform, with excellent specificity for the ELISA.

3.2. Results: detection of organisms

3.2.1. Detection of antigen in water samples by MEIA:

3.2.1.1. MEIA sensitivity and specificity. The sensitivity of the MEIA protocol was evaluated by determination of the *S. negevensis* IFU or number of amoebae present in the highest dilution of control material giving a positive signal. Using anti-*S. negevensis* serum diluted 1:20,000, 121 IFU of *S. negevensis* could be detected in the lysate and 468 infected amoebae could be detected. Uninfected *A. polyphaga* amoebae served as a negative control in the latter case. No signal was detected in the absence of anti-*S. negevensis* serum in the assay. When the same sample volumes were used for detection of amoebal antigen, the MEIA was able to detect 1000 amoebae. *S. negevensis*-containing cell culture lysates and a filter used in the assay without first antibody served as negative controls for the amoebal assay.

Figures 1 and 2 in the article attached (Kahane et al., AEM, June, 2004 70:3346-51) demonstrate the specificity of the MEIA assay for Simkania: Figure 1 with respect to a number of common bacterial respiratory pathogens, and Figure 2 with respect to members of the *Chlamydiaceae*. A volume of 100 microliters was used for the assay. None of the panel of standard bacteria gave a positive signal with antibodies to *S. negevensis* and affinity purified goat anti-rabbit HRP conjugates (Fig. 1), nor did *C. trachomatis* or *C. pneumoniae* antigens, under the same conditions (Fig 2).

3.2.1.2. Survival of *S. negevensis* in chlorinated and in distilled water. As described in our publication on the detection of Simkania in water (10), we determined that Simkania was able to survive chlorination treatment of water under conditions more stringent than those normally used for urban drinking water. Moreover, the organisms were able to survive in sterile distilled water for over a week, and even multiply if they were within amoebae (10). As shown in the table below, both *S. negevensis* and *Acanthamoeba* antigens were detectable in most samples of drinking water tested, which were obtained from homes in various neighborhoods of Beer Sheva.

Table 10. Detection of *S. negevensis* and Acanthamoeba antigens in samples of drinking water[a].

Neighborhood	No. positive samples / No. samples tested	
	S. negevensis antigen	*Acanthamoeba* antigen
1	12/12	12/12
2	3/3	3/3
3	5/5	5/5
4	1/3	3/3
5	0/3	1/3

[a]Samples (500 ml) were collected from a number of houses located in Beer Sheva on 3 different occasions. (30.1.03, 8.4.03, and 6.5.03) day 1, day 67 and day 95.

3.2.1.3. Detection of *S. negevensis* in drinking water and in reclaimed waste water. Simkania was detected in almost all samples of reclaimed waste water tested in the Negev, as shown in Table 11, and amoebal antigens were found in all such samples.

Table 11: The presence of Simkania (Sn) and amoebal (Am) antigens (as detected by MEIA) in samples of reclaimed waste water and the quality characteristics of the water[a].

Treatment type/ water quality[b]	Positive samples/tested samples		BOD range	COD range	(T)SS range
	Sn antigen	Am antigen			
Intensive/ good	7/7	6/6	9.5-26.1	28-62	5.5-26
Intensive/ poor	2/2	2/2	104-107	170-181	33-144
Extensive/ good	25/25	17/17	12.2-77.4	12.8-250	30-190
Extensive/ poor	5/6	4/4	86.5-208.1	220-610	63-297

[a]BOD: biological oxygen demand; COD: chemical oxygen demand; (T)SS: total suspended solids.

[b]Intensive, activated sludge;extensive, oxidation ponds.

In Peru, water samples obtained from the shanty-town area of San Juan de Miraflores were tested for the presence of S. negevensis by a protocol that was less sensitive than the final protocol adapted in Beer Sheva. The results are shown in Table 12. At the time of this writing, the most sensitive MEIA protocol is being used to test additional samples of water in Lima.

Table 12. The presence of Simkania antigens (as detected by MEIA) in samples of drinking water from several types of water sources, and stored for various periods of time before testing and in samples of waste water in Lima.

Water type / source	n	No. positive	% positive
Drinking water			
Well water	15	4	27
Cistern water	29	4	14
Tap water	13	0	0
Misc. or unspecified	20	5	25
Total drinking water	78	13	17
Sewage water	17	14	82

3.2.2. Results: detection of organisms by PCR

3.2.2.1. Water samples. In Beer Sheva, all samples positive by MEIA that were tested for the presence of Simkania DNA by PCR were positive. In Lima, problems were encountered with the PCR assay of drinking water, and only one sample was PCR positive for Simkania (It was not one of those positive by MEIA.) At this time, PCR assays are being carried out on fresh samples of water, with filters sent from Israel and purchased, according to the latest protocols, and with special attention being paid to possible pitfalls.

3.2.2.2. Nasopharygeal wash samples (NPW): NPS samples from children up to 3 years old with severe respiratory tract infection. These were tested by PCR for the presence of Simkania. Of 70 correctly obtained and stored samples taken from children hospitalized in the Dos de Mayo Hospital, none was positive by PCR for Simkania sequences. Of 14 such samples taken at the del Nino Hospital, none were positive, but of 49 samples taken and stored improperly, two were positive for Simkania sequences.

4. Summary and brief discussion

Severe respiratory illness is the number one cause of childhood mortality in Lima, Peru, and in most cases, the etiology is unknown. This study was initiated in order to begin to evaluate the contribution of *C. pneumoniae* and *S. negevensis* to severe respiratory illness in Peru, as well as to explore some aspects of the epidemiology of these infections. Such aspects included the prevalence of infection in various population groups, the age of acquisition of infection and potential modes of transmission. In contrast to the pathology of *C. pneumonia*e, which has bee extensively studied in Europe and in the United States, knowledge of Simkania pathology was rather limited. S. negevensis had been shown to be associated with bronchiolitis in children and with CAP in adults, and antibodies to this microorganism were found commonly in small population samples tested.

In this study, there were significant achievements at a number of levels:
a. Several new techniques were developed or adapted, including for detection of antibodies (dot blot and MIF) and for detection of microorganisms at the level of antigens (MEIA) and at the level of DNA (PCR).

b. *S. negevensis* was shown to be able to grow in free-living amoebae under laboratory conditions and survive within amoebal cysts. Techniques were developed for quantitative and qualitative detection of the microorganism within amoebae.

c. Evidence for the presence of *S. negevensis* or *S. negevensis*-like organisms in drinking water and in waste water was found both in the Negev and in Lima, Peru.

d. A high prevalence of antibodies to both *C. pneumoniae* and *S. negevensis* was found in children and some adults in Lima, Peru, and infection was acquired early in life; but while there was some evidence for a role for the first of these organisms in pediatric lower respiratory disease, that was not the case of *S. negevensis*. On the other hand, *S. negevensis*, but not *C. pneumoniae* seems to have a role in acute exacerbations of COPD in the Negev. Some evidence was found that in the Negev, infections with *S. negevensis* tend to occur during the winter months.

Further study of implications of these findings with respect to transmission of infection and methods of testing and treatment of water supplies is needed.

F. Impact, Relevance, and Technology Transfer

During the project, various protocols such as dot blot for serology, MEIA, and PCR techniques were shared with the group in Peru, which was updated whenever such a protocol was improved. Since the laboratory in Peru is not equipped to grow *S. negevensis* to purify antigens or DNA from locally grown organisms, various materials were transferred to Peru: filters impregnated with *S. negevensis* antigen for dot blot assays, rabbit polyclonal antibodies specific for Simkania, standards of Simkania DNA and amoebal control DNA, etc.

While severe respiratory infections with *S. negevensis* may not be common in Lima, such infections with *C. pneumoniae* may be more so. The pattern of acquisition of *S. negevensis* infection with age is not very clear yet and should be studied in additional population samples. Also, testing water with the best protocols developed in this study may reveal that more samples are positive for the organism than reported in this report. It may be that the Simkania-like microorganisms found in Peru differ from those found in Israel -- such a possibility can be examined in the future by sequencing of local isolates. Alternatively, it is possible that environmental conditions (pollution?) or water treatment policies differ in such a way that they do not allow a high prevalence of these bacteria. The technologies transferred to Peru, especially the serologic dot blot assay and the MEIA, are simple, require no sophisticated equipment, and can easily be adapted for the detection of other bacteria.

As more becomes known about Simkania and similar microorganisms, the techniques developed in the framework of this project and the experience of the Peruvian scientists with them, may become more and more useful.

It may be noted that with funds from this grant a 96-place manifold filtering device was purchased, and well as a pulse-oximeter for the children's hospital in Lima.

G. Project Activities/Outputs

1. Meetings held: It will be noted that more meetings should have been held, however, these did not come about for several reasons. The second Intifada and the second Gulf War contributed to a certain reluctance on the part of our Peruvian colleagues to come to Israel and to repeated postponements of planned trips. The planned trip to Peru by the Israeli PI did not take place because of a series of unforeseen family events, all between November 2003 and the beginning of March 2004. The trip could not take place during the second academic semester because of extensive teaching responsibilities. The alternatively suggested trip of young Peruvian scientists to Israel after the original official expiration of the grant (i.e., our request for an extension) was not approved.

a. In Lima, Peru, Dr. Simona Kahane met with Mrs. Pat Sheen, Prof. Bob Gilman, Mrs. Maritza Jimenez Calderone, Ms. Juliana Cordova and Ms. Fanny Arenas for discussions about their experiments, about the techniques developed in Israel (see below) and about our plans for future studies. (Feb. -Mar., 2002.

b. In Baltimore, MD, Dr. Simona Kahane met with Dr. Gilman (March, 2003) to discuss progress in the project, review plans and new protocols, and transfer to him new reagents for our colleagues in Peru.

c. In fall, 2003, Dr. Gilman came to Beer Sheva, to meet with Dr. Friedman, Dr. Kahane and Dr. Dvoskin. The achievements of the project were discussed, especially with respect to tfurther research in Peru, future publication of the results of the project. The possibility of sending Simkania-positive water samples from Peru to Israel in order to validate results found there and to attempt isolation of Simkania-like microorganisms was discussed. Isolation of such microorganisms could contribute significantly to the study of Simkaniae.

2. Training that occurred in the framework of the project.

a. During Dr. Simona Kahane's trip to Lima (in February, 2002) Maritza Jimenez Calderone and Juliana Cordova were trained in the performance of dot-blot assays. Also, Dr. Kahane was able to pinpoint problems in clinical sample collection and storage and offer concrete suggestions for improved collection and storage for the samples to be collected.

b. During July and August, 2003, Ms. Arenas was trained at Johns Hopkins at the Institute of Tropical Health and successfully completed the certificate course. Her training there was funded in part by this grant.

c. During the visit of Dr. Gilman to Beer Sheva (October 29-November 2, 2003), our new membrane technology was explained to him, for him to guide the Peruvian scientists in its application upon his return.

d. Other "training" and problem-solving sessions were held throughout the course of the grant via email, fax and telephone conversations.

3. Publications from the project (no patents):

a. Kahane, S, Dvoskin B, Mathias M, Friedman MG (2001). Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* survival within amoebal cysts. Appl. Environ. Microbiol. 67:4789-95.

b. Lieberman D, Dvoskin B, Lieberman DV, Kahane S, Friedman MG (2002). Serological evidence of acute infection with the chlamydia-like microorganism *Simkania negevensis* (Z) in acute exacerbation of chronic obstructive pulmonary disease. Eur. J. Clin. Microbiol. Infect. Dis. 21:307-9.

c. Friedman MG, Dvoskin B, Kahane S (2003). Infections with the chlamydia-like microorganism *Simkania negevensis*, a possible emerging pathogen. Microbes and Infection 5:1013-21.
d. Kahane S, Platzner N, Dvoskin B, Itzhaki A, Friedman MG (2004). Evidence for the presence of *Simkania negevensis* in drinking water and in reclaimed waste water in Israel. Appl. Environ. Microbiol. 70:3346-51.

H. Project Productivity

This project in some areas did not accomplish all the goals set, but in others, went beyond the original goals. While we did not develop a direct fluorescence assay for detection of *S. negevensis*, we did develop a simple membrane enzyme immunoassay (MEIA) that is much more suitable for developing areas. Furthermore, the principle involved can be applied to detection of any number of other organisms, the required reagents for that being high-titered, polyclonal antisera to the organism in question. We also developed a simple dot-blot serologic assay that is quite suitable for use in developing areas. We determined that exposure to *S. negevensis* or very similar organisms is fairly common in Lima, although perhaps not to the extent as in Israel and some other places in the world. Exposure to *C. pneumoniae* is even more common, and we found some serologic evidence indicating its possible involvement in severe respiratory tract disease in infants, although it was not detected much in NPS of ill infants. *S. negevensis* can be found in sewage water and some drinking water, and it may be found in respiratory samples from children with severe respiratory disease. However, from this study alone, one cannot conclude that it is a significant cause of such illness. Further studies are required to determine the degree of contribution of both agents to severe respiratory tract infection. This study has resulted in development of the tools needed for the accomplishment of such further studies.

During the course of this project, there should have been more training of young Peruvian scientists. Although materials can be sent by mail and courier, and communication by email and telephone can be efficient, there is no question that personal visits are most productive. We feel that the more personal involvement there is, the more commitment to the goals of the project; the personal interaction also leads to a greater determination to overcome practical difficulties. In the case of this project, the second Intifada and the second Gulf War led to a certain reluctance on the part of our colleagues to visit Israel. This reluctance is understandable and perhaps justifiable, but it did lead to fewer than optimal training opportunities.

I. Future Work:

The project can lead to future work of great significance, and our colleagues in Peru now have the ability to carry out many of these studies, either on their own, or in collaboration with other laboratories.

1. To determine whether Simkania is mainly a pathogen or a commensal bacterium. Are there long term effects of infection? What are the results of infection in immunocompromised hosts? Does primary infection with Simkania have any effect on the outcome of later infection with *C. pneumoniae*, for example?
2. Is water the main source of infection with Simkania?
3. It would be advantageous to obtain Simkania-like isolates from Peru. (This could have been done, but not within the original time-frame.) These would be interesting from a taxonomic and epidemiologic point of view. Since the full genome sequence of *S. negevensis* is now being determined, taxonomic comparisons will become relatively simple to achieve.

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Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* Survival within Amoebal Cysts

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Simkania negevensis*, a novel microorganism belonging to the family *Simkaniaceae* in the order *Chlamydiales*, has an intracellular developmental cycle during which two morphological entities, elementary bodies (EB) and reticulate bodies (RB), are seen by electron microscopy. Rates of seropositivity to the organism are high in certain population groups, and *S. negevensis* has been associated with respiratory illness in humans. This study reports for the first time the ability of *S. negevensis* to survive and grow inside *Acanthamoeba polyphaga* in addition to its known ability to grow in cell cultures of human or simian origin. Infectivity of *S. negevensis* and growth in amoebae were monitored by immunoperoxidase assays. Long-term persistence and exponential growth of *S. negevensis* in amoebal trophozoites were demonstrated by infectivity assays and by electron microscopy. EB and dividing RB of *S. negevensis* were observed within inclusion bodies inside *A. polyphaga*. When *S. negevensis*-infected *A. polyphaga* amoebae were exposed to adverse conditions resulting in encystation of the amoebae, several possible outcomes were observed: cysts containing both normal amoebic cytoplasm and *S. negevensis*; cysts in which *S. negevensis* cells were relegated to the space between cyst walls; and cysts containing *S. negevensis*, but apparently lacking amoebal cytoplasm. *S. negevensis* within dried amoebal cysts was capable of long-term survival. The possibility that amoebae may have a role in natural transmission of *S. negevensis* needs to be investigated.

Simkania negevensis is a recently discovered chlamydia-like intracellular microorganism (17, 18, 20) that has been associated with bronchiolitis in infants (19) and with community-acquired pneumonia in adults (23). Exposure to the organism, formerly referred to as "the microorganism Z" or "Simkania Z," is widespread in the Negev region of Israel (12), in Vancouver, British Columbia, Canada, in Brooklyn, N.Y., and in Lima, Peru (unpublished data).

S. negevensis has been phylogenetically assigned to a new family, *Simkaniaceae*, in the order *Chlamydiales*, based on ribosomal DNA (rDNA) sequence comparisons (8). The recently proposed reorganization of the taxonomy of the order *Chlamydiales* includes two other new families, *Waddliaceae* and *Parachlamydiaceae* (8, 28). Microorganisms of the latter family have been shown to be able to grow as endocytobionts in protozoa of the genus *Acanthamoeba* (1, 16), and evidence for their possible association with respiratory illness has been reported (5). Detection of parachlamydia-related 16S rDNA sequences in respiratory specimens and specimens of aortic tissue has also been reported (13, 27).

Free-living amoebae such as *Acanthamoeba* are commonly found in natural water sources and usually feed on bacteria; however, some bacteria, such as *Legionella pneumophila*, are capable of surviving within amoebae, amoebal cysts, or amoebal respirable vesicles, rendering the bacteria highly resistant to adverse conditions, such as elevated temperature, chlorination, and biocidal compounds (3, 21). At the molecular level, legionellae interact with their protozoan hosts much as they do with mammalian cells (14). In this study, we wished to determine whether *S. negevensis*, which was first discovered as a cell culture contaminant, is able to grow in trophozoites of acanthamoebae and survive within amoebal cysts.

MATERIALS AND METHODS

Growth of *S. negevensis*, standard purification procedure, and determination of IFU. Vero cells were grown in RPMI medium supplemented with 15% fetal calf serum, 1% glucose, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 1.2 μg of nystatin per ml, 8 μg of gentamicin per ml, and 50 μg of vancomycin per ml. *S. negevensis* (ATCC VR 1471T) was grown in Vero cell cultures in the presence of 1 μg of cycloheximide per ml. For the standard purification procedure, cultures were harvested with glass beads between 7 and 15 days after infection and mildly sonicated. Cell debris was removed by centrifugation for 10 min at 1,000 × *g*. *S. negevensis* reticulate bodies (RB) and elementary bodies (EB) were purified on Urografin (76%; Schering, AG, Berlin, Germany) gradients as described by Caldwell et al. (6) for purification of *Chlamydia trachomatis*. Briefly, after removal of cell debris by low-speed centrifugation, the bacterial suspension was centrifuged through a 35% (vol/vol) Urografin "cushion" (30 min at 54,000 × *g*) in a Beckman SW28 rotor. The pellet was resuspended in HEPES (25 mM)-saline buffer and layered onto a discontinuous (40% to 44% to 52% [vol/vol]) Urografin gradient in HEPES-saline buffer. After centrifugation for 45 min at 54,000 × *g*, RB and EB bands were removed by puncturing the gradient tube and diluted in HEPES-saline; particles were sedimented by further centrifugation (30 min at 54,000 × *g*). The number of infectious-center-forming units (IFU) was determined by 10-fold dilution and infection of Vero cells cultured in 96-well plates. Three days postinfection, the plates were fixed in 95% ethanol for 10 min at room temperature and examined for IFU by the microtiter plate immunoperoxidase assay (PIPA), performed as described previously (19). Briefly, after fixation, plates were incubated for 1 h at 37°C with *S. negevensis*-specific antisera raised in rabbits, washed, and reincubated (1 h, 37°C) with swine anti-rabbit horseradish peroxidase-conjugated antibodies, and, after another wash, stained with diaminobenzidine as a substrate. Infectious centers were counted under a magnification of ×200 with an inverted microscope and averaged for triplicate wells, and the number of IFU per milliliter in the original sample was calculated.

Cultivation of amoebae and estimation of number of amoebae in closed culture bottles. *Acanthamoeba polyphaga* strain Linc Ap-1, described by Fallon and

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Rowbotham (10) and kindly provided by R. J. Birtles (Unité des Rickettsies, CNRS EP J 0054, Faculté de Médecine, Marseille, France) was grown at 25°C under axenic conditions in PYG medium (32) supplemented with 100 U of penicillin ml^{-1}, 100 μg of streptomycin ml^{-1}, 1.2 μg of nystatin ml^{-1}, 8 μg of gentamicin ml^{-1}, and 50 μg of vancomycin ml^{-1}.

Growth of *A. polyphaga* in 25-cm^2 flasks (in 5 ml of PYG medium) was monitored by observation under an inverted phase-contrast microscope. The amoebae in 10 microscope fields (magnification, ×200) were counted, and the average number per field was calculated. Preliminary calibration of amoebal counts per microscope field against hemocytometer counts, by using uninfected amoebae, made determination of the number of amoebae in closed culture flasks possible. A calibration factor of 2.6×10^3 to 3.0×10^3 amoebae per ml for each amoeba seen in the field (at ×200 magnification) was established from eight separate calibration experiments. In this way, manipulation of infected amoebae was kept to a minimum.

Encystation procedure. The encystation procedure described by Sykes and Band (31) was used: *A. polyphaga* were grown in PYG for 3 days to 2×10^6 ml^{-1}, scraped with glass beads, and centrifuged for 10 min at 1,000 × *g*. The pellet was washed with a low-salt solution (50 mM NaCl, 4.6 mM $MgSO_4$, 0.36 mM $CaCl_2$) and resuspended to the original volume in high-salt solution (250 mM NaCl, 4.6 mM $MgSO_4$, 0.36 mM $CaCl_2$). The suspension was incubated for 40 h at 25°C, and the cysts were aliquoted, pelleted as described above, and washed with the high-salt solution. They were stored as a dry pellet either at 4°C or at room temperature.

Growth of *S. negevensis* in *A. polyphaga*. *A. polyphaga* was grown in 5 ml of PYG in a supine 25-cm^2 flask until the posterior wall of the flask was almost covered with organisms. This resulted in a total density of about 1.5×10^6 amoebae ml^{-1}. Growth medium was gently removed without disturbing the layer of amoebae, purified *S. negevensis* grown in Vero cells was added at a multiplicity of infection (MOI) of 1.0 IFU per amoeba in 1 ml of PYG, and the flask was incubated at 25°C for 2 h. Amoebae were gently displaced with glass beads (1 mm in diameter) and diluted to 30 ml, and the suspension was equally divided among three new flasks (time zero). For the growth curve, samples were taken at various times postinfection and frozen at −70°C in the presence of 50% fetal calf serum until assayed by titration on Vero cells.

Titration on Vero cells of IFU of *S. negevensis* from amoebae. Prior to titration, samples of frozen infected amoebae were diluted in RPMI infection medium containing 1 μg of cycloheximide ml^{-1}, which prevents *A. polyphaga* from feeding on Vero cells. Titrations were carried out in triplicate in 96-well microtiter plates, and 3 days later, the number of IFU was determined after staining of the wells in our standard PIPA (19).

Light microscopic detection of *S. negevensis*-infected *A. polyphaga*. An immunoperoxidase assay (IPA) for detection of *A. polyphaga* infected with *S. negevensis* in 25-cm^2 flasks was developed based on a similar assay used for cell cultures infected with *S. negevensis* (19). After the standard fixation in 95% ethanol for 10 min, additional treatment with a solution containing 90% methanol, 5% H_2O, and 5% H_2O_2 (30%) for 10 min was necessary in order to neutralize the endogenous peroxidase activity of the amoebae. The remainder of the procedure was identical to that for peroxidase-based detection of infected cell cultures.

EM of infected amoebae. Cultures of infected amoebae were pelleted, fixed, embedded in aralyte, and stained for electron microscopy (EM) as described by Biberfeld (4).

RESULTS

Infection of *A. polyphaga* trophozoites with *S. negevensis*. *A. polyphaga* trophozoites could be infected with *S. negevensis* in several ways: the amoebae could be added to infected Vero cell cultures, purified *S. negevensis* particles grown in Vero cells could be incubated with *A. polyphaga* in PYG medium, or persistently infected amoebae could be diluted in PYG in the presence of uninfected amoebae. The IPA was developed to stain *S. negevensis*-infected *A. polyphaga*, as shown in Fig. 1. Infected amoebae displayed one or more vesicles stained blue with antibodies specific for *S. negevensis*. Uninfected amoebae were not stained so long as endogenous peroxidase activity was neutralized after fixation, as described in Materials and Methods. The IPA was used for simple monitoring of the presence of *S. negevensis* in amoebal trophozoites.

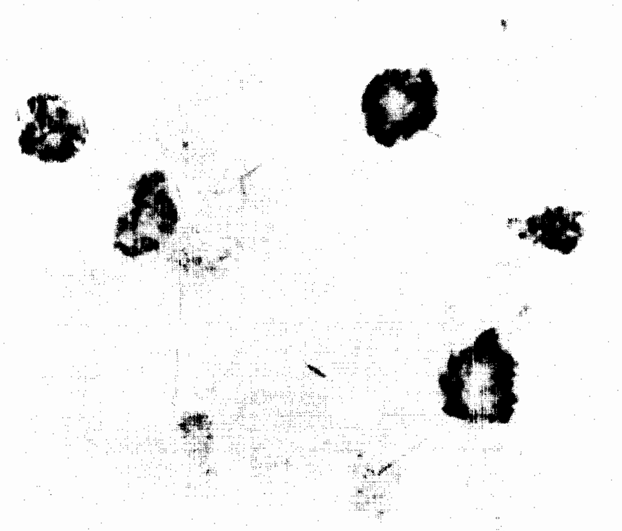

FIG. 1. Determination of percentage of acanthamoebae infected with *S. negevensis* by IPA. Infected amoebae stain dark blue with hyperimmune rabbit serum, peroxidase-conjugated swine anti-rabbit antibodies, and benzidine substrate. Stained and unstained amoebae are easily distinguished. Magnification, ×400.

To determine the morphology of *S. negevensis* in infected amoebae, *S. negevensis*-infected trophozoites of *A. polyphaga* were fixed and prepared for EM. Thin sections displayed inclusions containing one or more EB and RB particles of *S. negevensis* (Fig. 2). The inset in Fig. 2 shows two of the inclusions at higher magnification. The EB are somewhat elongated, with condensed chromatin, and the RB are rather pleomorphic. Such inclusions were observed in *A. polyphaga* axenic cultures even weeks and months after original infection, demonstrating the ability of *S. negevensis* to exist as endocytobionts of the amoebae.

The developmental cycle of *S. negevensis* in *A. polyphaga*. *A. polyphaga* amoebae were infected at an MOI of 1 with purified *S. negevensis* grown in Vero cells. The increase in the number of amoebae as well as the number of infectious *S. negevensis* particles (IFU, assayed on Vero cells) was monitored over time. Figure 3A shows survival of *S. negevensis* for 15 days without significant proliferation. The rather dense population of *A. polyphaga* continued to grow. When a sample from a 15-day flask from the experiment depicted in Fig. 3A was diluted 80-fold in fresh PYG, the increase in the number of amoebae as well as *S. negevensis* infectivity was monitored as a function of time, and the results shown in Fig. 3B were obtained. Exponential growth of both amoebae and the *S. negevensis* organisms infecting them was observed.

EM studies were carried out to characterize morphological features of the two types of infection represented by the growth curves of *S. negevensis* in Fig. 3. In the nonproliferative state (Fig. 3A), only a small proportion (<1%) of amoebae were seen to be infected. *S. negevensis* particles, mainly EB, were seen inside inclusions (usually containing only single particles), almost no dividing RB were seen, and mitochondria were not seen in the proximity of the inclusions (Fig. 4 A, C, and E). In contrast, in the "proliferative" mode (Fig. 3B), EB

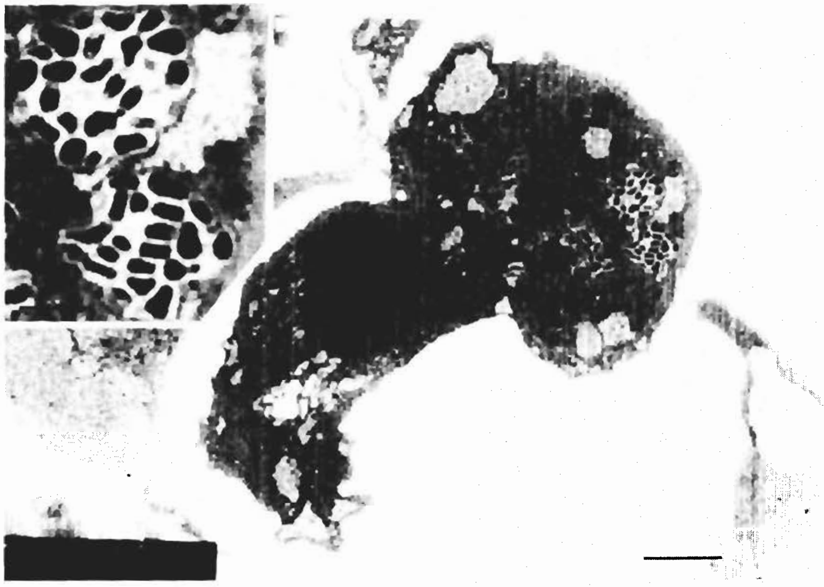

FIG. 2. Transmission electron micrograph of a thin section of an *A. polyphaga* trophozoite infected with *S. negevensis*. Inclusion vesicles containing numerous or single RB and EB are seen. Bar = 1 μm. (Inset) Enlarged portion of the figure showing two inclusions containing somewhat elongated, condensed EB and rather pleomorphic RB. Bar = 0.3 μm.

and dividing RB could easily be seen, even at 24 h postinfection (Fig. 4B). Later in infection, many *S. negevensis* inclusions were seen containing both EB and dividing RB particles (Fig. 4D and F). The inclusions were closely surrounded by a large number of host mitochondria.

Encystation of *S. negevensis* in *A. polyphaga*. The life cycle of *Acanthamoeba* spp. comprises two distinct stages: the trophozoite (vegetative, feeding form) and the cyst (dormant form, which develops under adverse conditions). The cysts are double-walled, resilient entities, which can survive exposure to temperatures between −20°C and +42°C (2). The fate of *S. negevensis* residing in *A. polyphaga* under conditions in which encystation occurred was examined by EM of thin sections of encysted material. Figure 5A shows a precyst in which a num-

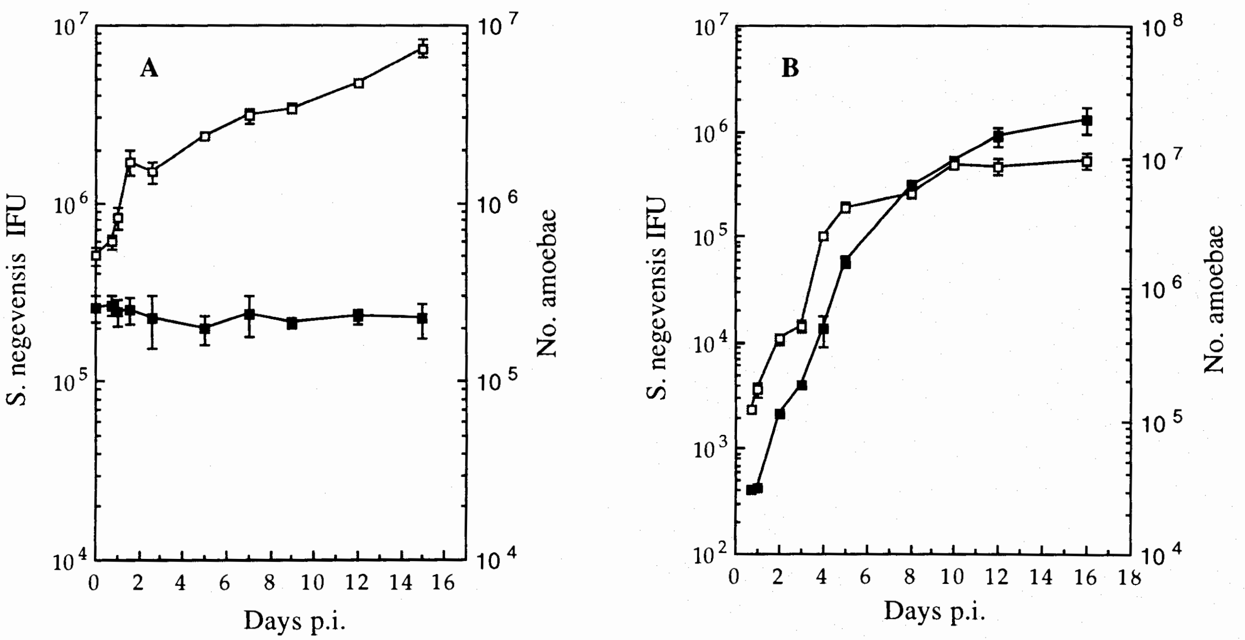

FIG. 3. Growth curves of *S. negevensis* in *A. polyphaga* and of the amoebae themselves in the same cultures. (A) Infection of a relatively high-density amoebal culture at an MOI of 1, with *S. negevensis* purified from infected Vero cell cultures. (B) Results after dilution of the 15-day culture shown in panel A into fresh PYG medium (see text). Solid symbols, IFU of *S. negevensis*; open symbols, number of amoebae in the culture at the given time point. Bars indicate the standard deviation of the mean for amoebal counts and *S. negevensis* titers determined in triplicate. p.i., postinfection.

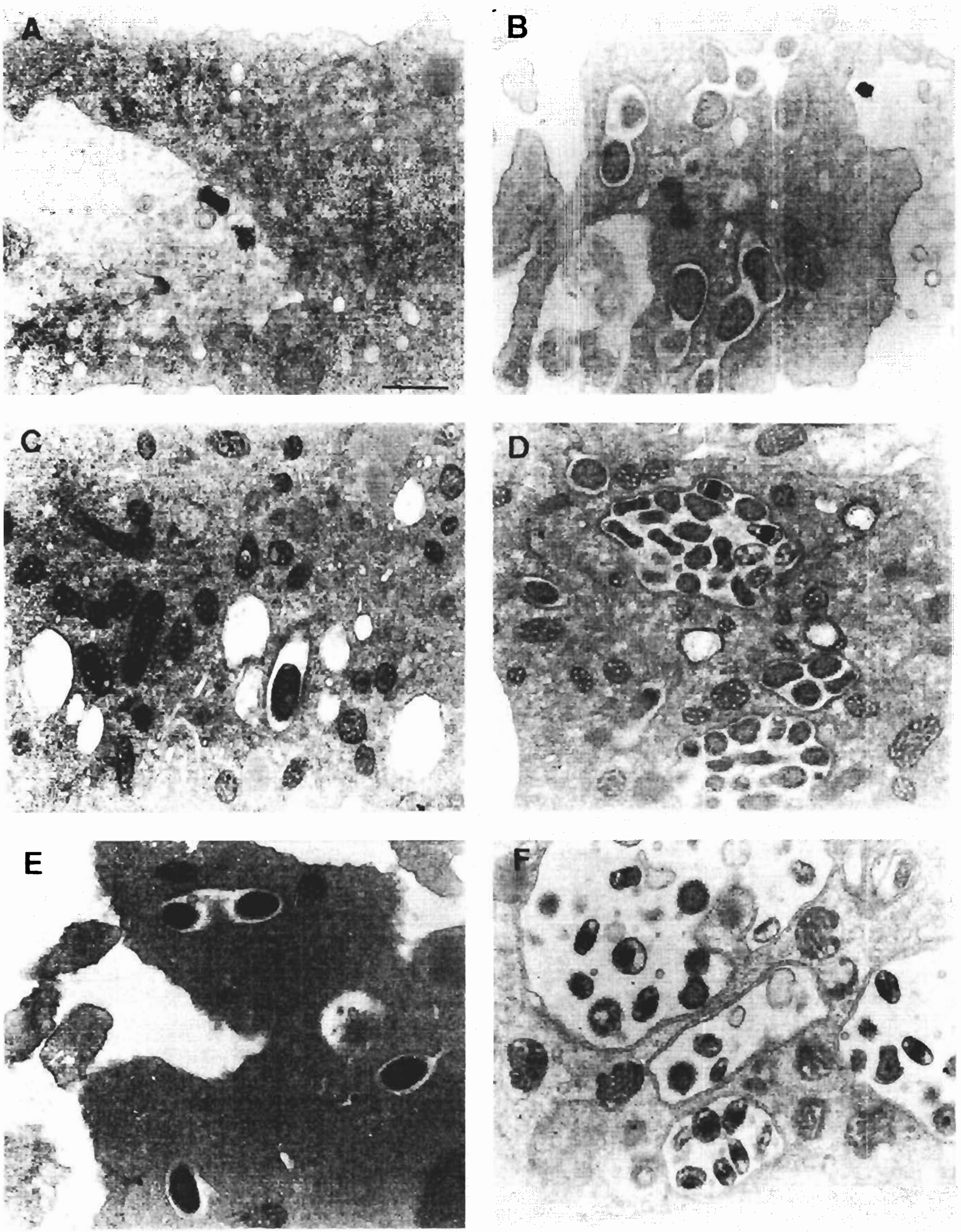

FIG. 4. Time course of *S. negevensis* nonproliferative infection (left panels) versus productive infection (right panels) of amoebae, as monitored by EM. (A, C, and E) One, 7, and 15 days postinfection, respectively. (B, D, and F) One, 10, and 16 days postinfection, respectively. Bar = 0.5 μm.

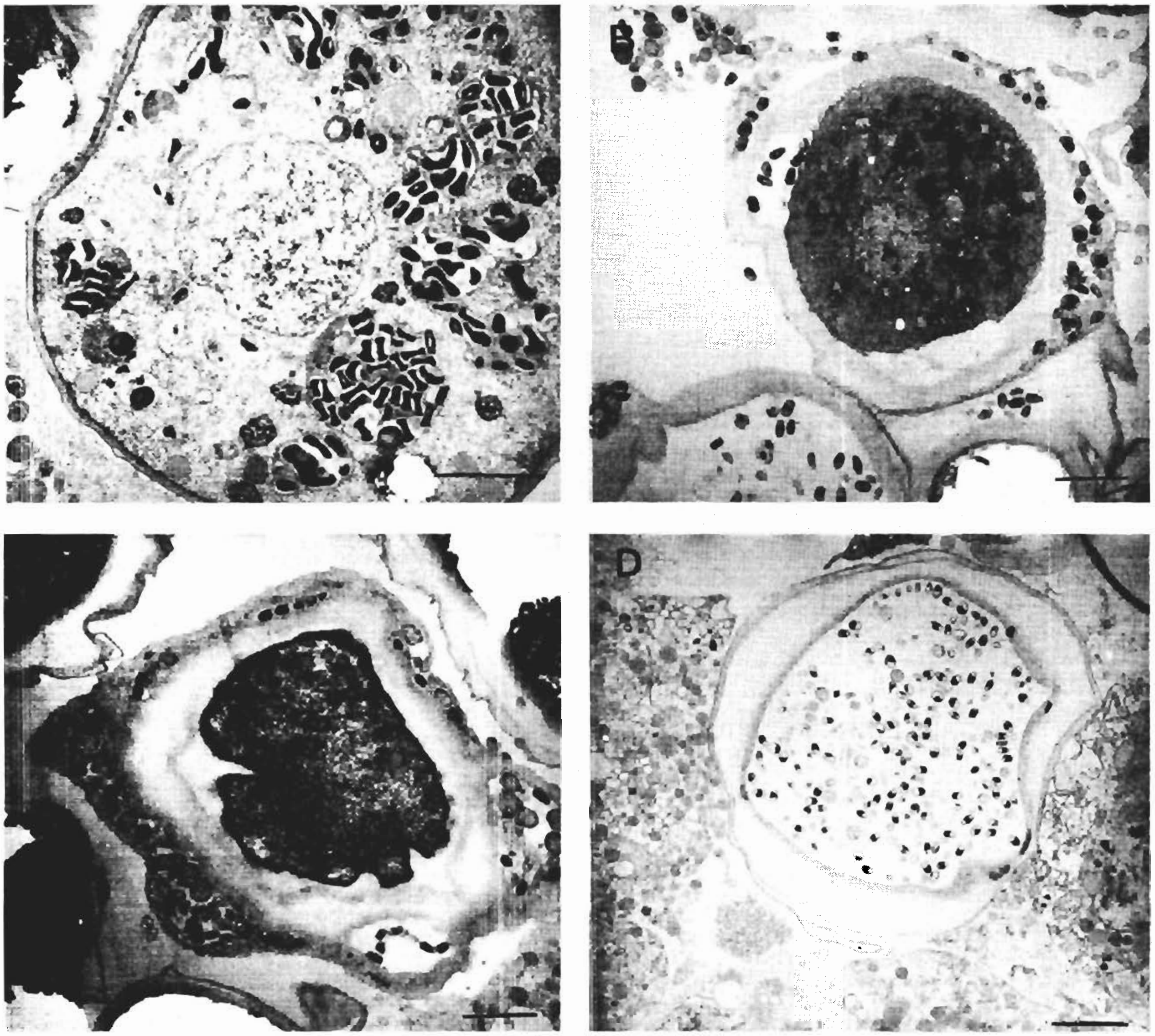

FIG. 5. Electron micrographs of *A. polyphaga* infected with *S. negevensis*, 2 days after encystation, demonstrating several possible interactions between the two organisms. (A) Precyst. (B to D) Double-walled cysts. Bars = 1 μm.

ber of *S. negevensis* inclusions containing both EB and RB particles are seen. In the process of encystation, when the double wall was formed, *S. negevensis* particles could be seen between the cyst walls or on the inner side of the outer wall (Fig. 5B and C). In Fig. 5B, some bacteria could still be seen residing inside the cyst cytoplasm, while in Fig. 5C, it appears as if the amoeba succeeded in excluding *S. negevensis* from the central cytoplasm of the cyst. However, another outcome was observed (Fig. 5D) in which only the bacteria seemed to reside inside the double wall of the cyst. In parallel with the EM studies, cysts were prepared and stored dry at room temperature or at 4°C. The ability of *S. negevensis*-infected cysts and uninfected cysts to convert to trophozoites upon return to optimal growth conditions was observed by microscopy, and the recovery of *S. negevensis* infectivity was monitored by PIPA (Table 1). After 79 days at 4°C, infectivity of *S. negevensis* that had been sequestered in amoebal cysts was still greater than 50% of initial infectivity, while purified *S. negevensis* EB particles did not survive 12 days of exposure to room temperature or 4°C, even when they were suspended in SPG (sucrose-phosphate-glutamic acid medium), a standard storage solution

TABLE 1. Survival of *S. negevensis* in cysts of *A. polyphaga* as a function of time[a]

Time (days)	Infectivity (% of initial) at:	
	Room temp	4°C
0	2.5×10^6 (100)	2.5×10^6 (100)
21	2.13×10^6 (85)	2.6×10^6 (100)
48	2.23×10^6 (89)	ND[b]
79	ND	1.4×10^6 (56)
148	7.75×10^3 (0.3)	8.6×10^3 (0.3)

[a] Results of a representative experiment are shown. Other experiments gave similar results, but the longest time point has not yet been repeated.
[b] ND, not determined.

used to preserve the microorganisms at −70°C (data not shown).

DISCUSSION

S. negevensis was initially isolated as a contaminant of cell cultures, and its natural host or hosts were unknown. However, the finding that its 23S rRNA contains an unspliced group I intron similar to those found in chloroplasts of algae and mitochondria of amoebae (9) stimulated investigation of its ability to infect amoebae. In this study, the ability of *S. negevensis* to grow in *A. polyphaga* as well as in cultured cells of human or simian origin was demonstrated for the first time. Infection of *A. polyphaga* with purified *S. negevensis* grown in Vero cells resulted in loss of most of the bacterial infectivity; however, in some fraction of the amoebal culture, *S. negevensis* was able to survive for more than 15 days and eventually multiply (Fig. 3). A similar phenomenon was observed when *Acanthamoeba castellani* amoebae were infected with *Chlamydophila pneumoniae* (7), an intracellular microorganism of the *Chlamydiaceae* family that causes epidemic and endemic respiratory infections in humans and has been associated with chronic and acute cardiovascular disease. Dilution of amoebae persistently infected with *S. negevensis* and their transfer to favorable nutritional conditions allowed exponential growth of *S. negevensis* (Fig. 3B). By EM, EB and dividing RB particles could be seen inside very distinct inclusions, which were closely surrounded by a large number of mitochondria (Fig. 4B, D, and F); these images were very similar to those seen in infected HeLa cells (17) and Vero cells (unpublished results). EB were 0.2 to 0.3 μm in diameter and usually contained some white, electrolucent areas in addition to areas of condensed DNA. RB were more pleomorphic than EB, with dimensions of 0.3 to 0.7 μm, and had no areas of condensed DNA.

Encystation of *S. negevensis*-infected amoebae under unfavorable environmental conditions resulted in competition for survival between *S. negevensis* and the amoebae. Several possible outcomes were observed: (i) amoebal cysts in which *S. negevensis* was found between the cyst walls (Fig. 5B and C), as was previously described by Steinert et al. (30) in the case of *Mycobacterium avium*; (ii) cysts in which both *S. negevensis* and normal amoebic cytoplasm were seen; and (iii) cysts in which *S. negevensis* remained alone, protected by the double wall of the cyst (Fig. 5D). Similarly, legionellae have been shown to be able to survive and be transmitted inside amoebal cysts (21, 29; reviewed in reference 15).

Free-living protozoa such as *Acanthamoeba* are commonly found in water supplies, air, desert dust, and cooling systems (24, 25). These organisms have been associated with amoebic keratitis, especially in wearers of contact lenses (24), and can also produce a rare encephalitic infection in immunocompromised individuals. Many types of bacteria have been found within free-living amoebae, including *Legionella* spp., *Burkholderia pickettii*, *Vibrio cholerae*, *Mycobacterium avium*, and *Listeria monocytogenes* (26, 30; reviewed in references 14 and 15). These bacteria are able to survive inside the protozoa as endocytobionts and to take advantage of them as vectors for their dissemination as pathogens.

A variety of chlamydia-like microorganisms have recently been detected by PCR in diverse clinical specimens and environmental samples (27). Additional endocytobionts belonging to the order *Chlamydiales* as determined by amplification of 16S rDNA are being recovered from clinical and environmental isolates of *Acanthamoeba* spp. and *Hartmannella vermiformis* (13, 16). The survival of *S. negevensis* as an endocytobiont in trophozoites of *A. polyphaga* and in cysts demonstrated in this study may have implications for the mode of transmission of the microorganism. Although we have shown preliminary evidence for the survival of *S. negevensis* in amoebal cysts for as long as 21 weeks (Table 1), the potential for survival under various types of adverse conditions still needs to be systematically examined. The widespread seropositivity to the organism in different population groups (11, 12) and the apparently early age of acquisition of infection, at least in some population groups (11; M. G. Friedman, A. Galil, D. Greenberg, S. Greenberg, R. Dagan, B. Sarov, and S. Kahane, Abstr. First Cong. Eur. Soc. Emerg. Infect., Budapest, Hungary, poster 15, 1998), support the possibility that amoebae may have a role in the natural transmission of *S. negevensis*, since, in the past, early acquisition of infection with *Helicobacter pylori* was associated with transmission in drinking water (22).

It has been suggested that microorganisms capable of surviving and growing inside amoebal hosts may have gained some evolutionary advantage allowing growth and survival within human macrophages, since in some ways, living protozoa mimic professional macrophages (13). The ability of *S. negevensis* to grow exponentially both in protozoa and in cell culture implies that this microorganism may be useful as a model system for comparison of the physiology of mechanisms of survival in different hosts and has important implications for transmission and pathogenesis of the microorganism.

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CONCISE ARTICLE

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Serological Evidence of Acute Infection with the Chlamydia-Like Microorganism *Simkania negevensis* (Z) in Acute Exacerbation of Chronic Obstructive Pulmonary Disease

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Abstract The aims of this study were twofold: (i) to test for possible associations between serological evidence of acute *Simkania negevensis* (Sn) infection and acute exacerbation of chronic obstructive pulmonary disease and (ii) to examine the prevalence of past infections with Sn in patients with chronic obstructive pulmonary disease. In 120 patients (63%) there was serological evidence of past infection with Sn, which was not significantly different from the rate in a control population. In five hospitalizations serological evidence existed of acute infection with Sn around the time of the exacerbation of chronic obstructive pulmonary disease. In four of these cases, there was serological evidence of acute infection with at least one other respiratory pathogen. It is concluded that Sn can be associated serologically with exacerbation of chronic obstructive pulmonary disease, in most cases together with other respiratory pathogens. The implications of these findings should be investigated further.

Introduction

Simkania negevensis (Sn), previously known as the *Chlamydia*-like microorganism Z, is a recently discovered intracellular bacterium that was first thought to belong to the family *Chlamydiaceae* [1]. More recent evidence indicates that it belongs to a separate family in the order *Chlamydiales* [2]. Its replication cycle seems to be similar to that of Chlamydia in that it is characterized by the appearance of electron-dense elementary bodies and dividing reticulate bodies [3]. However, while *Chlamydia* species have 95.5% 16S ribosomal DNA identity among themselves and 73% identity with certain rickettsia such as *Cowdria ruminantium*, *Ehrlichia anaplasma*, and *Coxiella burnetii*, the known *Chlamydia* species have only 80–87% identity with Sn [4].

From a clinical standpoint, infection with Sn is common in the general population of southern Israel [5], and it may be associated with bronchiolitis in infants [6] and with community-acquired pneumonia (CAP) in adults [7]. Given the association between Sn and CAP in adults, it is logical to question whether an association also exists between Sn and other respiratory tract infections among adults.

Within the framework of a comprehensive study of the infectious etiologies of patients hospitalized for acute exacerbation of chronic obstructive pulmonary disease (AECOPD), a database was created for a large number of hospitalizations and a broad range of infectious etiologies, which were identified by means of innovative and sensitive serological techniques [8]. Using the sera and clinical data that were collected in that study, the objective of the present study was to test for possible associations between serological evidence of acute Sn infection and episodes of AECOPD. An additional objective of the study was to examine the prevalence of past infections with Sn among patients with chronic obstructive pulmonary disease (COPD) and to compare it with parallel prevalence in a non-COPD population.

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Materials and Methods

All hospitalizations for AECOPD during the period between 1 November 1997 and 15 March 1999 in the internal medicine and intensive care wards of the Soroka Medical Center in Beer-Sheva, Israel that met the inclusion criteria and whose patients gave consent to participate were included in the study.

Inclusion criteria for the study hospitalization group were all of the following five conditions: (i) patient age more than 40 years; (ii) chronic airway obstruction as determined by spirometry; (iii) a smoking history of at least 20 pack years; (iv) at least one of the following complaints in the week before hospitalization: increased shortness of breath, significant increase in sputum production, new expectoration of purulent sputum or increased sputum purulence; and (v) lack of pneumonia in an admission chest radiograph. The control population included 100 trauma and rehabilitation patients with age and gender distributions identical to the COPD patients. The details of the study protocol have been described previously [8].

All sera were tested for antibodies to Sn and to *Chlamydophila (Chlamydia) pneumoniae* using in-house enzyme-linked immunosorbent assay tests as described previously [7]. A level of ≥0.60 adjusted optical density (OD), which was well above the gray level of 0.4–0.5 OD, was taken as evidence of past infection with Sn. Current/acute infection was indicated by a changing level of ≥0.5 OD units for Sn-specific IgA and/or IgG between paired sera. In none of 200 paired control bank samples was a difference greater than 0.41 adjusted OD units found (between paired samples).

In this study the work-up for detection of other infectious etiologies was based exclusively on serological testing. We described the methods, kits, and criteria used for these serological diagnoses in a previous publication [8].

Results and Discussion

Two hundred seventeen AECOPD hospitalizations of 190 different patients were included in the study population for the diagnosis of acute Sn infection. A convalescence serum sample was obtained in all these 217 hospitalizations at a mean of 24.3±5.4 days (range, 17–53 days) after the first sample was collected at the beginning of the hospitalization. The mean age of the 190 patients was 67.2±8.7 years; 161 (85%) were male, and 70 (37%) were current smokers. The mean age of the 100 control patients was 67.1±10.8 years; 84 (84%) were male, and 27 (27%) were current smokers (no significant difference with the COPD group for any of these three variables).

Serological evidence of past infection with Sn (in the acute-phase serum sample in the 190 single or first hospitalizations) was found in 120 COPD patients (63%) and in 69 control patients (69%) (not significant). In five AECOPD hospitalizations (2.3%) there was serological evidence of acute Sn infection around the episode. Patient characteristics of these five hospitalizations, including date of hospitalization and other infectious etiologies identified, are presented in Table 1. At least one other infectious pathogen was found in four of these five patients.

Table 2 details specific serologic test results for each of the paired sera in each of the five hospitalizations for both Sn and *Chlamydia pneumoniae*. Results are also given for two additional hospitalizations for two of the patients that either came before or followed the index hospitalizations and were included among the 217 AECOPD hospitalizations in the study. No significant changes in *Chlamydia pneumoniae*-specific IgG or IgA antibodies were found for any of these five hospitalizations.

Although a small percentage of episodes of AECOPD are apparently not caused by infection, many of the episodes with classic infectious features are still categorized as being of unknown infectious etiology. Some of these episodes may be caused by as yet unrecognized respiratory pathogens. The results of the present study provide support for this possibility.

The proof of association between episodes of AECOPD and Sn in this study is based on serological testing and not on isolation or identification of pathogens in respiratory tract secretions. Besides the technical difficulty in isolating and/or identifying Sn in respiratory tract secretions, we have no convincing proof that the presence of the pathogen in the respiratory tract provides strong evidence of a significant association between it and the acute disease. This assertion is particularly true in light of the high prevalence of past infections with this pathogen in the general population. In contrast, a significant change in the Sn-specific antibody level between acute- and convalescence-phase sera does provide evidence of a significant association between the pathogen and the patient, which occurred around the time of the episode. Support for this contention can be gained from our finding that no significant change was found in two of the five patients in two other hospitalizations for AECOPD as described in Table 2. Although it is clear today that the microorganism Sn does not belong to the family *Chlamydiaceae*, it has a closer phylogenetic relationship to this family than to other microorganisms [1]. Thus, we found it appropriate to present the results of serological tests for *Chlamydia pneumoniae* in these five patients. None of these patients had a significant antibody response to this pathogen around the time of the acute exacerbation (Table 2).

The prevalence of past infection with Sn was not significantly different between the COPD and control populations. This finding provides evidence that this pathogen is common in the general population and not only among COPD patients. The past infection rate of

Table 1 Characteristics of the five patients with AECOPD and serological evidence of acute infection with *Simkania negevensis*

Patient no.	Date of hospitalization	Age (years)	Sex	Additional infectious etiologies
1	12/1/98	72	M	none
2	18/1/98	65	F	influenza virus type A
3	26/1/98	71	M	*Mycoplasma pneumoniae*, *Legionella gormanii*, *Haemophilus influenzae*, influenza virus type A
4	31/1/99	55	M	*Streptococcus pneumoniae*
5	20/2/99	66	F	*Mycoplasma pneumoniae*, parainfluenza virus type 1

Table 2 Comparison of enzyme-linked immunosorbent assay (adjusted OD 492 nm) antibody levels to *Chlamydia pneumoniae* and *Simkania negevensis* in paired serum samples of the five patients with serological evidence of acute infection with *Simkania negevensis*

Patient no.	Serum sample	*Chlamydia pneumoniae*		*Simkania negevensis*	
		IgG	IgA	IgG	IgA
1	1	0.236	0.274	2.017	0.230
	2	0.216	0.223	1.460	0.209
	1[a]	0.254	0.240	1.166	0.310
	2[a]	0.232	0.284	1.117	0.286
2	1	0.264	0.205	1.376	0.438
	2	0.196	0.177	0.665	0.302
3	1	0.289	0.577	0.658	0.347
	2	0.274	0.548	1.388	0.314
4	1	0.367	0.361	1.803	0.474
	2	0.292	0.287	0.961	0.302
	1[b]	0.322	0.254	0.535	0.142
	2[b]	0.343	0.283	0.619	0.189
5	1	1.176	0.513	1.500	0.525
	2	0.857	0.495	0.793	0.470

[a] Parallel serological data for an AECOPD-related hospitalization 254 days after the index hospitalization
[b] Parallel serological data for an AECOPD-related hospitalization 334 days before the index hospitalization

63–69% among COPD and control patients is within the range of 55–80% prevalence found in the general population of our region in a previous study [5].

Three of the five patients with serological evidence of infection with Sn around the time of the acute episode were hospitalized within a period of 14 days. This aggregate of three patients within a short period of time is of special interest. The three patients lived in three different settlements and there was no contact among any of them before hospitalization. It is also unlikely that the pathogen was transmitted through patient-to-patient contact during hospitalization, since they were hospitalized in three different hospital wards. It is noteworthy that in the series of CAP patients with serological evidence of Sn infection that we reported on in the past, there was a similar group of four patients who became ill within a period of 1 month [7].

In four of those five patients at least one other respiratory pathogen was also identified that was serologically associated with the acute episode. This high percentage of patients with serological evidence of respiratory infection with more than one pathogen is well established for CAP, especially in association with atypical pathogens [9]. We also described this condition in respiratory tract infections in the general practice setting [10] and among the AECOPD patients included in the present study [8]. An additional respiratory pathogen was found in four of the eight CAP patients with evidence of acute Sn infection [7]. Taken together, these data point to the conclusion that, much like other atypical respiratory pathogens, Sn is prevalent in polymicrobial respiratory infections. Since the diagnoses are based on serological testing, we cannot determine whether the Sn infection came first and facilitated the penetration of other pathogens or came in the wake of the other pathogens. Similarly, the role of Sn in terms of the clinical manifestations of the disease remains unclear.

We conclude that Sn can be associated serologically with AECOPD, in most cases together with additional respiratory tract infections. The clinical and therapeutic implications of our findings should be investigated further.

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Review

Infections with the chlamydia-like microorganism *Simkania negevensis*, a possible emerging pathogen

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Abstract

Although evidence for the existence of numerous chlamydia-like microorganisms has been discovered in both environmental samples and clinical specimens, very few have been grown in vitro, and little is known of their pathogenic potential. Of all such organisms, *Simkania negevensis* is probably the most extensively studied. This review summarizes current knowledge about this intracellular bacterium, focusing especially on human infections.

Keywords: Communicable diseases, emerging; Bacteriology; Disease transmission

1. Introduction

Simkania negevensis is an intracellular bacterium that was first detected when rapidly moving particles were observed in cytoplasmic inclusions in laboratory stocks of Vero cells. Transmission electron microscopy of thin sections revealed small, dense forms and larger, homogenous forms, similar in appearance to chlamydial elementary and reticulate bodies. Studies were undertaken to determine the phylogenetic relationship of the organism to chlamydia and other intracellular bacteria and to characterize growth of the microorganism in cell culture. Techniques were developed for its detection in clinical specimens, and in parallel, serologic techniques were developed for detection of specific antibodies. The purpose of this review is to summarize briefly information on the phylogeny and growth and development of the organism in vitro, to review in detail current knowledge of human infections, and to suggest directions for future research.

2. Background on *S. negevensis*

2.1. Morphology and growth characteristics in vitro

S. negevensis (formerly called "Z" or "Simkania Z") was early on shown to be similar to the chlamydiae in a number of characteristics, but different in others. Two developmental forms were described, a homogeneously staining replicating form, resembling reticulate bodies of all known chlamydial species, and a more compact form, with both electron dense and electron lucent areas [1,2] (and see Fig. 1). These forms were slightly larger than the corresponding forms of *Chlamydia trachomatis*, strain L2. In contrast to what is known for chlamydial reticulate bodies, evidence was presented indicating that the replicating bodies of *S. negevensis* are infec-

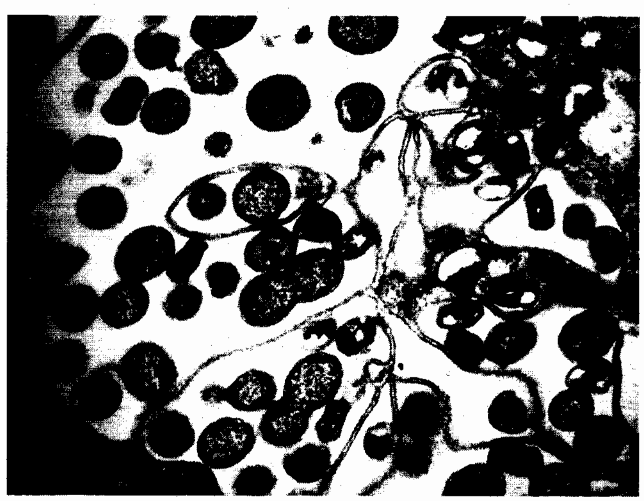

Fig. 1. Thin-section electron micrograph of a Vero cell 4 d after infection with *S. negevensis*. Dividing reticulate-like bodies as well as elementary-like bodies and intermediate bodies are seen. Bar on lower left: 0.5 μm. Cells were prepared for electron microscopy as described previously [3].

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tious [3]. The possible pathologic or epidemiologic significance of this finding is unclear. The growth cycle of the organism in vitro also differs from that of most of the chlamydiae in that after a 2–4-d period of exponential growth, the organism enters a plateau stage during which there is no increase in the number of infectious particles; however, cytopathic effects in infected cells continue to develop for up 2 weeks or more, as has been described and discussed previously [1–3]. Most chlamydiae lyse their host cells after 2–5 d of exponential growth.

2.2. Taxonomic relationships

S. negevensis belongs to a new family, Simkaniaceae, in the order Chlamydiales [4] based on its obligately intracellular parasitic growth in cultured cells, a biphasic morphology of electron dense and reticulated forms, size comparable to that of all known chlamydiae, and rDNA sequence comparisons. The *S. negevensis* full-length 16S and 23S rDNA sequences are each 80–87% identical to those of members of the Chlamydiaceae, whereas all members of the Chlamydiaceae have >90% identity with each other. The genome length of *S. negevensis* is 1.7 Mbp (mega base pairs), compared to 1.1–1.2 Mbp for members of the family Chlamydiaceae [2] and 1.6 Mbp for UWE 25, which belongs to another new family in the Chlamydiales, the Parachlamydiaceae []. For a full discussion of the revised chlamydial taxonomy and the three new families in the order Chlamydiales, see the web site: http://www.chlamydiae.com.

Although the full genome sequence of *S. negevensis* has not been determined, partial DNA sequences of the RNase P gene (*rnpB*), *mutS*, and *groEL* have been determined from PCR-amplified products (Genbank accession numbers AF000190, AF468693, and AY219919, respectively) using primers based on consensus sequences determined from several strains of chlamydia and other bacteria. Taxonomic relationships among Chlamydiaceae species have been confirmed by analyses of GroEL (the 60-kDa heat shock protein (Hsp60)), KDO transferase, the small cysteine-rich lipoprotein, the 60-kDa cysteine-rich protein, and the major outer membrane protein (MOMP) [5]. The cysteine-rich proteins and MOMP are unique to the chlamydial lineage. Thus, it may be that such sequences will be of value in the confirmation of proposed relationships between newly described Simkania-like microorganisms [6] and *S. negevensis*.

2.3. Antigens and membrane structure

The polypeptide pattern of *S. negevensis*, as shown by SDS PAGE, is significantly different from that of other members of the Chlamydiales [1]. Only a relatively small number of common epitopes (most likely heat shock proteins) are shown to be shared when antigen panels of *S. negevensis, Waddlia chondrophila, Parachlamydia acanthamoebae, C. trachomatis* and *Chlamydophila* (*Chlamydia*) *pneumoniae* are tested for cross-reactivity by western blot using specific polyclonal antisera [7]. Immunoblot analysis of Urografin-purified *S. negevensis* whole cell lysates and sarcosyl-insoluble membrane preparations using hyperimmune murine sera showed the following predominant membrane fraction antigens: a set of three bands migrating at 37–42 kDa, additional bands at 26, 52, 58, and 64 kDa, and a slower migrating band at >100 kDa. Monoclonal antibodies (MAbs) prepared against whole organisms or whole cell lysates reacted mainly with the 37- to 42-kDa set of bands, indicating that the complex was strongly immunogenic (unpublished). Some of these MAbs were capable of neutralizing *S. negevensis* in vitro, but were unable to neutralize *C. trachomatis* (L2 strain) under the same conditions. When these MAbs raised against *S. negevensis* were tested by immunoblotting on *C. trachomatis* whole cell lysates or membrane preparations, they were able to recognize an epitope located on the OmpA protein (unpublished). On the other hand, MAbs recognizing family-specific epitopes of chlamydial OmcB (OMP-2), OmpA (MOMP), and LPS did not bind *S. negevensis* antigens [1,2]. The structure and function of the three polypeptides belonging to the *S. negevensis* outer membrane complex need to be further investigated. It may be that the shared epitopes constitute part of a porin structure common to the Chlamydiaceae and *S. negevensis*.

2.4. Antibiotic and cytokine sensitivities

Although in its sensitivity to macrolides and tetracyclines, *S. negevensis* is similar to the chlamydia, unlike these cousins, it is totally resistant to penicillin, and, indeed, is routinely grown in the presence of penicillin. While penicillin and other beta lactams are not the drugs of choice for treatment of chlamydial infections, in vitro the organisms are sensitive to the presence of these drugs, and their development is inhibited at the reticulate body stage in the presence of beta lactams. *S. negevensis* was found to be resistant to bacitracin, but sensitive to D-cycloserine; both these drugs are inhibitors of peptidoglycan synthesis. Hybridization experiments using cloned *C. trachomatis* penicillin-binding protein DNA as probes (PBP-1, PBP-2, and PBP-3; a generous gift of C. Storey) indicated the presence of homologous DNA in *S. negevensis*. However, in preliminary experiments using tritiated penicillin (as described by Barbour et al. [8]), only one penicillin-binding protein (MW 46 kDa) was observed (unpublished results). Whether *S. negevensis* "penicillin-binding proteins" are not transcribed or have no penicillin-binding sites still remains to be investigated. Preliminary experiments indicate that azithromycin, minocycline and erythromycin are the most effective inhibitory and bactericidal drugs in cycloheximide-treated Vero cells. Doxycycline and ofloxacin are moderately inhibitory and bactericidal, while ciprofloxacin is less effective [9].

We have recently shown that *S. negevensis* is resistant to high concentrations of interferon gamma (up to 400 units/ml) when grown in HEp-2 cells (unpublished results), while the growth of most strains of *C. trachomatis* is inhibited by this effector cytokine [10]. On the other hand, *S. negevensis*

31

growth is inhibited by concentrations of tumor necrosis factor (TNF)-alpha that were shown to inhibit the growth of *C. trachomatis* L2/434/Bu (unpublished and [11]). These preliminary results are interesting, since we have also shown that *S. negevensis* can grow quite well in tryptophan-starved cells, indicating that the organism may be capable of synthesizing its own tryptophan. In any case tryptophan starvation cannot be the basis for its sensitivity to TNF-alpha. The possible pathologic consequences of interferon resistance are not clear, but see Section 7.

3. Detection of past and present infection with *S. negevensis*

Several methods for detection of *S. negevensis* infection have been developed, for the most part in our laboratory; methods developed in other laboratories have not yet been published in detail.

3.1. Detection of past infection by serology

An ELISA assay originally developed to distinguish between antibodies to *C. trachomatis* and *C. pneumoniae* [12] was modified for the specific detection of antibodies to *S. negevensis* [13]. For this assay, gradient-purified *S. negevensis* particles are used as antigen. The high specificity of the assay was achieved by the use of a 6 M urea wash step following incubation of antigen-coated plates with the serum samples being tested. The urea wash, which was followed by a standard washing procedure with PBS-Tween, released antibodies which were not of high affinity to the antigen. Thus, antibodies which were actually raised against a different, cross-reacting antigen were released in this step. Therefore, this ELISA assay, when used to detect IgG, is an effective screening test for antibody indicative of past infection with the organism.

3.2. Detection of current or recent infection

3.2.1. Serology

Serologic diagnosis of current or recent infection with *S. negevensis* was usually based either on a significant change in antibody level between acute and convalescent serum samples or on detection of serum IgA antibodies or both [14–16]. Assays were performed by ELISA for detection of IgG or IgA antibodies as above, or by the "IPA" immunoperoxidase technique, in which a mixture of infected and uninfected cells served as antigen; the mixture was applied to glass slides in drops, dried, and fixed [15]. In the IPA technique, results were determined by light microscopy in comparison to control positive and negative sera included in each test. It has been the experience of the authors that the ELISA test for IgA is not suitable for testing sera of very young children, while the IPA test is suitable for all sera, regardless of the age of the subject.

3.2.2. Detection of the organism in clinical specimens

For culture, 200-µl samples were used in duplicate to infect Vero cell cultures grown in 96-well plates. Infected cultures were passaged twice without dilution, at 7- to 10-d intervals. On the third passage, infected samples were diluted 1:2 and applied to two fresh plates: one plate served to detect *S. negevensis* by immunoperoxidase assay using hyperimmune rabbit serum; the second plate was used for further propagation of the organism from positive specimens [15].

For PCR testing, DNA was prepared from aliquots of the nasal wash specimens by the QIAamp blood kit (Qiagen, Hilden, Germany). DNA was concentrated by ethanol precipitation and amplified with forward and reverse primers from the 16S ribosomal DNA sequence of the organism, resulting in a 398-base pair amplicon. The forward primer begins in a region unique to organisms of the order Chlamydiales. *C. trachomatis, C. pneumoniae* and *C. psittaci* as well as rickettsiae, *Mycoplasma* species and *E. coli* did not amplify with the primers used [15].

4. Seroprevalence of antibodies to *S. negevensis*

4.1. Seroprevalence in adult population samples

A number of population samples have been tested for antibodies to the organism. In the Negev region of Israel, between 55% and 80% of several groups of healthy adults were seropositive to *S. negevensis* by the ELISA assay (see Table 1) [13]. The lowest seropositivity rates were among the non-commercial blood donors and the highest among Bedouin adults who accompanied others to the hospital. Interestingly, the seropositivity rates to *C. trachomatis* in these same serum specimens were rather low. In most of the groups, seropositivity to *C. pneumoniae* was similar to that

Table 1
Seropositivity to *S. negevensis*, to *C. pneumoniae*, and to *C. trachomatis* by ELISA assay

Group	*n*	Percent seropositive on different antigens [a]		
		S. negevensis	*C. pneumoniae*	*C. trachomatis*
Students	94	65	60 (n.s.)[b,c]	17 ($P < 0.001$)[b,c]
Blood donors	100	55	73 ($P < 0.05$)[b,c]	22 ($P < 0.001$)[b,c]
Kibbutz members, 18–40 years	106	64	58 (n.s.)[b,c]	17 ($P < 0.001$)[b,c]
Bedouin, 18–40 years	45	80	27 ($P < 0.001$)[b]	4 ($P < 0.001$)[b]

[a] Adjusted OD > 0.500 for *S. negevensis*; adjusted OD > 0.400 for Chlamydiae.
[b] Significance of seropositivity difference compared to *S. negevensis*; n.s., not significant.
[c] Significantly different from the Bedouin seropositivity rate for the same antigen.

32

for *S. negevensis*, except among the Bedouin, where it was significantly lower (Table 1).

It has become clear that infection with *S. negevensis* is not limited to the southern part of Israel. When serum samples of healthy adults from different parts of the world were tested for the presence of IgG antibodies to *S. negevensis* by the ELISA assay, the results shown in Table 2 were obtained [17]. While in some locations, past exposure to *S. negevensis* seemed to be more prevalent then to *C. pneumoniae*, in others the opposite was true. However, it appears that infection with the organism (or very closely related organisms) is rather common in many parts of the world.

4.2. Age of acquisition of infection

An appreciation of the age of acquisition of infection with a microorganism can be obtained by cross-sectional serum sampling for determination of seroprevalence in different age groups. Such studies were carried out with the entire population of a Negev kibbutz, who had blood samples drawn for determination of seropositivity to measles virus in the face of an impending outbreak in the area ($n = 290$). Fig. 2 shows the distribution of seropositivity by age for this group, with respect to *S. negevensis, C. pneumoniae*, and *C. trachomatis*. It is notable that infection with both *S. negevensis* and *C. pneumoniae* was acquired very early. Fig. 3 shows similar data for a sample of healthy Negev Bedouin of various ages ($n = 198$). In both groups, infection with *S. negevensis* was acquired at a very early age; however, among the Bedouin, infection with *C. pneumoniae* was acquired much more slowly (or perhaps the organism was more prevalent several decades ago than more recently). Kibbutz children are in close contact with their peers from a very early age, whereas most Bedouin children begin organized activities in groups when they start school at age 6. The different pattern of acquisition of infection with the two microorganisms may reflect differing modes of transmission of infection (see also Section 6).

5. Pathology associated with infection with *S. negevensis*

5.1. Respiratory tract

Several associations of *S. negevensis* with respiratory tract disease have now been described. The organism has been associated with bronchiolitis in infants by PCR, culture, and serology [15]. In that study, there was a significant difference between the number of patients vs. controls positive for organism detection and a significant difference between the number of patients and controls positive for *S. ne-*

Table 2
Seropositivity to *S. negevensis* (*Sn*) and *C. pneumoniae* (*Cpn*), as determined by ELISA, in healthy adults in various parts of the world

Location	Subjects	*n*	*Sn* (%)	*Cpn* (%)
Vancouver, Canada	First trimester pregnant women	100	68	35
Brooklyn, NY	Healthy adults aged 19–72	42	39	51
Beer Sheva, Israel	Orthopedic ward patients	200	68	78
Aarhus, Denmark	Blood donors, aged 19–63	106	65	Nd[a]

[a] Nd, not done.

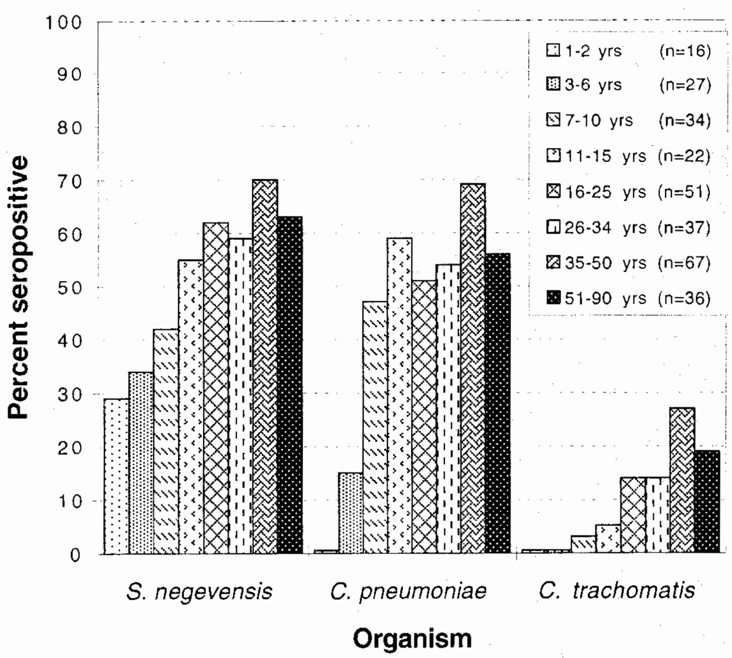

Fig. 2. Seropositivity to *S. negevensis, C. pneumoniae* and *C. trachomatis* by age in residents of a Negev kibbutz, as determined by ELISA assay.

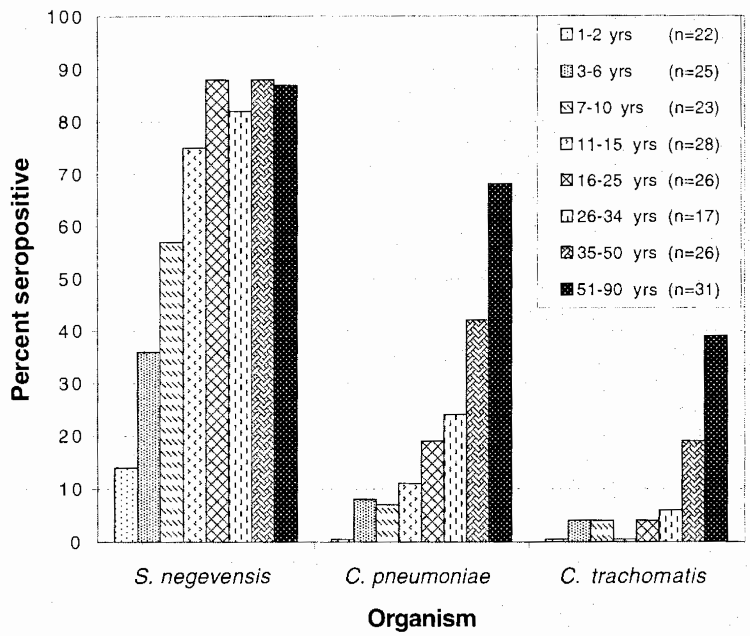

Fig. 3. Seropositivity to *S. negevensis*, *C. pneumoniae* and *C. trachomatis* by age in healthy Bedouin of the Negev, as determined by ELISA assay.

gevensis-specific IgA by serology by IPA (see Table 3). Detection of the organism in nasopharyngeal washes was by PCR and culture. Of the 34 positive patient specimens, 13 were positive by both PCR and culture, 3 by culture alone, and 18 by PCR alone. When bronchiolitis patients were divided into three groups (infection with respiratory syncytial virus alone, infection with *S. negevensis* alone, or infection with both) and clinical findings were compared, no significant differences were found. Nor were there any significant differences in demographic variables (age, sex, ethnic origin, type of dwelling, and number of family members) among these three groups [15]. The data described in that study indicated an association of *S. negevensis* with bronchiolitis; however, infection with the organism did not seem to increase the degree of morbidity in respiratory syncytial virus-infected patients, and therefore its possible presence as a commensal agent could not be completely excluded.

In another study, *S. negevensis* was found to be highly prevalent in Inuit infants hospitalized with bronchiolitis on Baffin Island, Nanuvet, Canada. Fourteen of 22 nasal washes obtained from such infants were PCR positive for the organism (D. Greenberg et al., Scand. J. Infect. dis. (in press)). Severe lower respiratory tract infections are extremely common in this population of indigenous children, who have a hospital admission rate for such disease of 306/1000 during the first year of life. In the study reported here, 10 of the children were intubated and transferred to a tertiary center for treatment. One of these, infected only with *S. negevensis*, required mechanical ventilation for 17 d. Other etiologies sought in this study were seven respiratory viruses, *C. pneumoniae* and *C. trachomatis*. Clearly, further studies are needed to corroborate these findings; at least one such study is currently under way.

Table 3
Detection of *S. negevensis* in nasopharygeal washes and serologic reaction to infection with *S. negevensis* in patients with bronchiolitis and in controls

Parameter	Patients	Controls	*P*
Positive for organism detection	34/120 (28%)	0/56	<0.001
Positive for IgA by serology	14/92 (15%)	1/78 (1%)	

The possible association of *S. negevensis* with community-acquired pneumonia (CAP) and with acute exacerbation of chronic obstructive pulmonary disease (AE-COPD) in adults is based on serology alone. It has been argued that serology is a more accurate reflection of acute infection than is culture or PCR detection of an organism in nasopharyngeal swabs (NPS) or other secretory samples; especially in adults, a number of potentially pathogenic microorganisms may colonize the respiratory tract without necessarily being involved in the current illness. In a study of 308 patients with CAP from whom paired serum samples were available, and in which 13 possible etiologies were sought, eight patients had serological evidence of acute infection with *S. negevensis*. CAP was defined by the presence of an acute febrile disease, acute pulmonary infiltrate on chest radiogram, and a clinical and radiological course that confirmed the diagnosis [14]. The criteria used to determine association with infection with *S. negevensis* were very high ELISA IgA levels (OD > 1.2) or increasing IgG or IgA levels between paired sera (difference in OD ≥ 0.5 between paired samples). Among the eight patients, none had evidence of current infection with *C. trachomatis* or *C. pneumoniae*, and all eight were hospitalized between mid-February and mid-June, although the study ran through a calendar year. In four of the eight, between one and three additional etiologic agents were diagnosed, but in the other four, no other potential etiologic agent could be identified. The latter four patients were all about 30 years old, and three had no history of chronic illness. Their current illness was characterized by high fever and a non-productive cough; three had pleuritic pain and three had gastrointestinal symptoms. All showed a prompt response to erythromycin therapy [14].

An association of *S. negevensis* with some cases of AECOPD was found in an intensive study of patients hospitalized with AECOPD during a 17–month period that included two winter seasons [16]. Criteria for inclusion in the study were all of the following five conditions: (i) patient age over 40 years; (ii) chronic airway obstruction as determined by spirometry; (iii) a smoking history of at least 20 pack years (one "pack year" being one pack of cigarettes per day for a year); (iv) at least one of the following complaints in the week before hospitalization: increased shortness of breath, a significant increase in sputum production, new expectoration of purulent sputum or increased sputum purulence; and (v) lack of pneumonia in an admission chest radiograph. In this study, 190 patients with a total of 217 hospitalizations were examined for serologic evidence for various etiologies of the exacerbation. Evidence for past infection with *S. negevensis* was found in 63% of these patients and in 69% of 100 control trauma and rehabilitation patients. Among the AECOPD patients, evidence for involvement of *S. negevensis* was found in five (2.3%). In four of the five, between one and four additional infectious etiologies were found as well. Three of the five became ill within a period of 14 d, although there was no contact among them before hospitalization, and they were hospitalized in three different hospital wards. The authors concluded that much like other atypical respiratory pathogens, *S. negevensis* is present in polymicrobial respiratory infections.

An association of *S. negevensis* infection with persistent cough without pulmonary disease has been reported in Aarhus, Denmark [18]. Paired serum samples of 79 patients (aged 16–74) with chronic cough of 2–12 weeks duration and an additional eight single serum samples were available, as were 106 single serum samples from healthy blood donors, aged 19–63. Serologic testing with the ELISA previously described showed no dynamics of OD levels between paired serum samples, possibly indicating that no acute infections were taking place. (OD readings of 89% of sample pairs were within 0.100 OD units of each other). However, while seropositivity rates for IgG were similar for patients and controls (49% and 65%, respectively), only 3% of controls, but 11% of patients with chronic cough had IgA antibodies to *S. negevensis* (Fisher's exact test, $P = 0.03$). Since no patients with 2–3 weeks' duration of cough were IgA positive, the rate of IgA seropositivity was 15% for patients with 4–12 weeks' duration of cough, resulting in a statistically significant association between chronic cough and the presence of IgA antibodies against *S. negevensis*. Other variables investigated and found to be without significant association with seropositivity to *S. negevensis* were smoking, sex, and age of the patients.

Petrich et al. [19] developed a PCR assay for amplification of a sequence from the *hsp-60* gene of *S. negevensis*. They used this assay to test peripheral blood mononuclear cells (PBMC) from 100 patients with clinically stable COPD for the presence of *S. negevensis* DNA (in comparison with *C. pneumoniae* DNA), and found 22% positive (compared to 24% for *C. pneumoniae* DNA). Although there was a trend toward more sputum production in *S. negevensis*-infected patients than others, the only parameter differing significantly between *S. negevensis*-infected patients and others was the season of the sampling, with *S. negevensis* infection associated with the winter season (November–April), odds ratio (95% confidence interval) of 4.8 (1.7–13.6), $P = 0.003$. The prevalence of *S. negevensis* in PBMC of healthy individuals also needs to be examined.

NPS specimens from 99 infants (<12 months of age) with respiratory tract illness and from 95 nursing home patients during influenza season were also tested by the PCR of Petrich et al. [19] for the presence of *S. negevensis* DNA. Four percent of the infants' samples were positive, as were 5.3% of the samples from the nursing home patients. The corresponding prevalences of infection with *C. pneumoniae* were 9.8% and 2%. The prevalence of *S. negevensis* in NPS samples from the healthy population should also be examined.

5.2. Other anatomical sites of infection

Whether *S. negevensis* infections are limited to the respiratory tract is unclear, but there are very preliminary indications that the organism may be found in the cardiovascular system. *S. negevensis* genome sequences were amplified from sections of carotid artery tissue (S. Montenegro, personal communication). Interestingly, *S. negevensis* has been shown to grow rather efficiently in U937 cells, a monocyte/macrophage cell line (Fig. 4). In the case of *C. pneumoniae*, it has been shown that infected macrophages may transmit the organism to vascular endothelial cells [20] and that in a mouse model, systemic dissemination of the organism can be achieved via infected macrophages [21].

6. Mode of transmission of infection in man

The rather young age of acquisition of infection with *S. negevensis* raises the question of the source of the infection. In the past, early age of infection with *Helicobacter pylori* was associated with its transmission in drinking water [22]. Since some newly described members of the Chlamydiales were in fact discovered as endosymbionts (endocytobionts) of free-living amoebae, the ability of *S. negevensis* to replicate in amoebae was also examined. It was found that not only were the organisms able to replicate in *Acanthamoeba polyphaga*, but they were also able to survive over long periods in amoebal cysts [23]. Since free-living amoebae such as acanthamoebae may be found in many water sources, as well as in desert sand [24], it may be that the

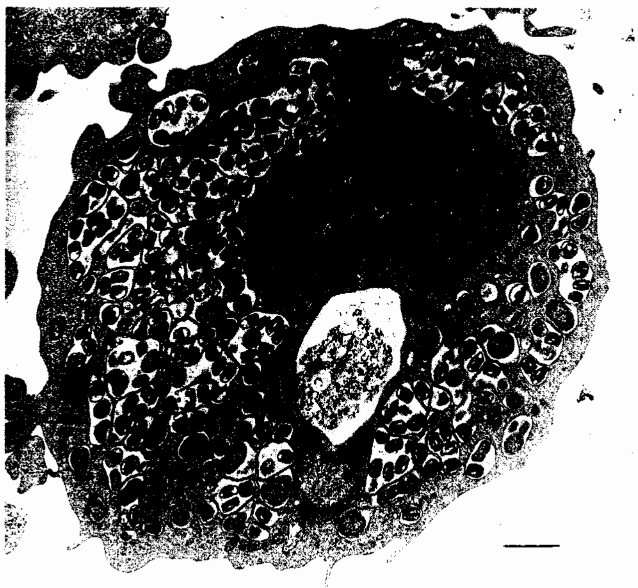

Fig. 4. Electron micrograph of a U937 macrophage 7 d after infection with *S. negevensis*. Multiplicity of infection: 0.07, bar = 1 µm. Cells were prepared for electron microscopy as described previously [3,23].

natural source of transmission of *S. negevensis* to man is via such amoebae. Similar transmission of *Legionella* has been described [25], and indeed amoebae are thought to play an important role in the natural history of infections with legionellae and possibly other intracellular microorganisms [26]. Such a mode of transmission would result in infection of children at a very young age, irrespective of whether they have close interactions with other children (e.g. day care centers or kibbutz children's houses). It may be noted that although it was possible to infect amoebae with *C. pneumoniae*, such infection did not result in rapid proliferation of the microorganism, but rather very slow growth [27]. Although the subject needs to be examined more carefully, at present it seems unlikely that *C. pneumoniae* is transmitted via water supplies. That may not be the case for other chlamydia-like microorganisms, such as members of the Parachlamydiaceae, many of which were first detected as endosymbionts of free-living amoebae.

7. Directions for future research

There has been an intriguing description of the finding of rDNA sequences closely related to *S. negevensis* sequences in various environmental and anatomic sites ([6] and M. Horn, Parachlamydiaceae, http://www.chlamydiae.com/chlamydiae/docs/Chlamydiales/family_parachlamydiaceae.htm, May, 2003). One area of research which is thus far unexplored is the variety of Simkania-like organisms that may exist. How closely are they related to each other? Do the serologic tests currently in use recognize antibodies to all the types? We have several isolates and are looking forward to examining some from different parts of the world as well. Can the organism be found in drinking water or in waste-water? The former would imply possible transmission via drinking water, while the latter would imply possible excretion from the digestive tract. We are in the process of developing a simple assay system for the detection of the organism in such samples, and preliminary indications are that it can be found in most waste-water samples tested and in some drinking water samples. Because of the ubiquity of free-living amoebae such as acanthamoebae, it may be worthwhile to test samples of desert sand or examine air samples in different locations.

One most interesting question is the nature of the inclusion vesicle in which this intracellular bacterium resides. On the one hand, like organisms of the genus *Chlamydophila* of the Chlamydiaceae, it seems to develop in multiple intertwining inclusions [1–3] and therefore may also lie on or just off the exocytic vesicle pathway, as has been described for *C. trachomatis, C. pneumoniae*, and *C. psittaci*. On the other hand, within the family Parachlamydiaceae, bacteria have been described (*Neochlamydia hartmannellae*) which actually seem to replicate free in the cytoplasm of infected amoebae, rather than within membrane-bound vesicles [28], as has been the case for all members of the Chlamydiales described until very recently. Whether the nature of the inclusion vesicle and the intracellular site of replication may have implications for pathogenesis is totally unknown.

A novel approach to learning more about the potential pathogenesis of *S. negevensis* is to examine in vitro which types of epithelial cells it is able to infect, and which of these it may activate to inflammatory cytokine secretion. Likewise, it is not yet known if it activates macrophages when it infects them (Fig. 4), and whether it is capable of infecting and activating endothelial or synovial cells. The answers to these questions will have implications for pathology since, for example, it has been shown that while *C. trachomatis* can infect endothelial cells (as can *C. pneumoniae*), it does not activate them to cytokine secretion, while infection with *C. pneumoniae* does [29]. It is *C. pneumoniae*, which for many other reasons is suspected of being involved in atherosclerosis, not *C. trachomatis*. Such studies should be able to indicate which clinical syndromes often of unknown etiology (such as arthritis and gastrointestinal tract inflammation) would be worthwhile to examine for possible involvement of *S. negevensis*.

Much of the damage caused by chlamydial infections (blinding trachoma, tubal infertility, and reactive arthritis) is thought to be due to the existence of the organism in a quiescent persistent state, in which it expresses significant quantities of chlamydial heat shock protein 60 and down-regulates expression of the MOMP. Such a state can be induced in vitro by a number of factors, including certain antibiotics, such as penicillin, and cytokines such as interferon gamma. Since *S. negevensis* is resistant both to penicillin and to interferon gamma, it is possible that it will be found to be less of a pathologic problem in terms of chronic infections. On the other hand, it is not yet known what the significance might be of the long plateau stage in its growth cycle. Are persistent forms of the organism created during the long plateau stage? Or might the extended growth cycle be due to long-term suppression of host cell apoptosis? The chlamydiae seem to be able to inhibit apoptosis, but also to induce it under certain conditions, especially toward the end of their growth cycle [30]. This is another area that needs to be explored with respect to *S. negevensis*.

Sequencing of the *S. negevensis* genome would furnish the opportunity for manifold comparisons to sequences of Chlamydiaceae genomes. Of special interest would be drug resistance and sensitivities, enzymes involved in metabolism, and membrane structure. Clearly, there is room for research in many directions. The results cannot be predicted, but since the organism seems to be ubiquitous, and since the research may have implications for pathogenesis of other organisms in the order Chlamydiales as well, it should be allocated a high priority.

8. Conclusion

Natural infection with *S. negevensis* seems to be highly prevalent, with infection being acquired at an early age, at least in some population groups. Current evidence indicates a possible role for the microorganism in respiratory tract infections, although there may be additional sites of infection as well. Transmission is probably by droplet or close contact, but there is some evidence that water supplies may also be implicated, and studies to determine whether that is the case are currently under way. Intracellular bacterial pathogens may have a variety of relationships with their hosts, leading either to acute or to chronic infections, and resulting in short-term or long-term pathologies. In chlamydial infections, the long-term sequelae of chronic infections are not directly proportional to the virulence of the initial infection, and initially mild disease may lead to significant damage. In view of the high seroprevalence of infections with *S. negevensis*, its potential pathogenic interactions with its host merit further investigation.

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Evidence for the Presence of *Simkania negevensis* in Drinking Water and in Reclaimed Wastewater in Israel

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***Simkania negevensis* is a recently discovered chlamydia-like intracellular microorganism which has been associated with bronchiolitis in infants and with community-acquired pneumonia in adults; a high seroprevalence of antibodies to the microorganism has been found in various population groups. *S. negevensis* can be grown in various cell lines as well as in free-living amoebae such as *Acanthamoeba polyphaga*. In this study, evidence for the existence of *Simkania* or *Simkania*-like microorganisms in drinking water and in reclaimed wastewater is presented for the first time. Detection of the microorganism was made possible by the development of a specific and sensitive filter membrane immunoassay and was confirmed by PCR detection of microbial DNA in the water samples. The common presence of *S. negevensis* in water sources together with the high seroprevalence of antibodies to it and early age of acquisition of infection may implicate water as a source of infection. The possible significance of this finding for public health and for municipal water testing and treatment needs to be further examined.**

Simkania negevensis is an intracellular bacterium which has been shown to be similar to chlamydiae in some of its characteristics (13, 14, 16). Taxonomic studies have placed *S. negevensis* in a new family, *Simkaniaceae*, in the order *Chlamydiales* (2). This bacterium has been associated with several types of respiratory tract disease including bronchiolitis in infants (by PCR, culture, and serology) (15) and community-acquired pneumonia and acute exacerbation of chronic obstructive pulmonary disease in adults (by serology) (20, 21, 23). Natural infection with *S. negevensis* seems to be highly prevalent. In the Negev region of Israel, for example, between 55 and 80% of several groups of healthy adults had serum antibodies against the organism (6). However, infection with *S. negevensis* is not limited to southern Israel, and it now appears that infection with this organism (or very closely related ones) is rather common in many parts of the world (5).

The seroprevalence of *S. negevensis* has been studied in groups of people of different ages, such as the entire population of a Negev kibbutz (n = 290) and healthy Bedouins of various ages (n = 198). The distribution of seropositivity by age indicated an early exposure to the infectious agent (5) and raised the question of the source of infection and mode of transmission. In the past, early age of acquisition of infection with *Helicobacter pylori* was associated with its presence in drinking water (18).

Since some newly described members of the *Chlamydiales* were in fact discovered as endosymbionts (endocytobionts) of free-living amoebae, the ability of *S. negevensis* to replicate in amoebae in the laboratory was also examined. It was found that not only were the organisms able to replicate successfully in *Acanthamoeba polyphaga* but they were also able to survive over long periods of time in amoebic cysts (12). In addition, other chlamydia-like microorganisms, such as members of the family *Parachlamydiaceae*, were shown to be able to grow successfully as endocytobionts of free-living amoebae (9, 11); some other *Chlamydiales* members, such as *Chlamydophila* (*Chlamydia*) *pneumoniae*, may infect these organisms but grow only slowly (1). Since free-living amoebae such as acanthamoebae may be found in many water sources, as well as in desert sand (22), it may be that the natural source of transmission of *S. negevensis* to humans is via such amoebae. Similar transmission of legionellae has been described previously (4), and indeed, amoebae are thought to play an important role in the natural history of infections with legionellae and possibly other intracellular microorganisms (10).

In this study, we examined the question of whether *S. negevensis* can be found in drinking water or in wastewater in the Negev. The development of a simple assay system for detection of the organism in such water samples is described, and evidence for the presence of *S. negevensis* in various water sources is presented.

MATERIALS AND METHODS

Water sources. Wastewater in the Negev (southern Israel) is treated intensively (activated sludge) or extensively (oxidation ponds) and is routinely evaluated for quality by the regional public health laboratory. The parameters measured include the biological oxygen demand, the chemical oxygen demand, and total suspended solids. Forty samples of reclaimed wastewater were obtained from the regional public health laboratory after their routine testing was completed but without our knowledge of the results of this testing until our experiments were complete. These were samples of water that had not received tertiary treatment (which would have included sand filtration and chlorine treatment, followed by testing for coliforms).

Samples of tap water were taken from several neighborhoods in Beer Sheva, which obtains its water supply from both the national water carrier and from deep wells, that is, underground water reserves (aquifers) located in the area. The supply alternates between these two sources but not in a set pattern. Drinking water samples were also obtained from three different satellite communities near Beer Sheva, which receive their water from local deep wells. Drinking water brought to the area by the National Water Carrier normally has a turbidity

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39

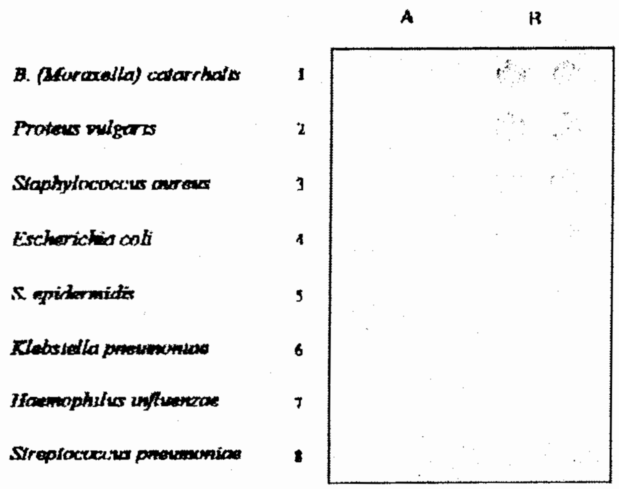

FIG. 1. Specificity of MEIA with respect to other bacteria commonly found in the respiratory tract. The MEIA was performed as described in Materials and Methods. Column A, 100 μl of various bacteria filtered in duplicate; column B, 100 μl of *S. negevensis* suspension in twofold dilutions.

measurement of <1 while the turbidity measurement of deep well water is usually <0.3. Drinking water is chlorinated to 0.1 to 0.5 ppm (or milligrams/liter) of free chlorine. Drinking water in the city is tested according to a municipal plan. In 2003, 684 tests were carried out. In one, 5 coliforms were detected, but upon retesting the source, none were found. All other samples were negative for coliforms.

Chlorination of water and determination of the survival of *S. negevensis* in chlorinated water. Chlorine levels in water were determined by a pocket calorimeter (HACH Europe, S. A./N.V., Floriffoux, Belgium) used for routine field testing of chlorine levels. Sterile deionized water (containing 0.07 mg of free chlorine/liter) was exposed for 20 min to chlorination (0.5 mg of free chlorine/liter), generated with free chlorine reagent powder (cat. no. 21055-69; HACH) which was later neutralized with sodium thiosulfate (1 M). *S. negevensis* particles or *S. negevensis* in persistently infected amoebae precipitated in a microcentrifuge were suspended in chlorinated water immediately after its chlorination treatment and incubated at room temperature (RT). Control tubes received the same water without chlorine treatment. At the beginning of the incubation, the chlorine levels ranged from 0.48 to 0.52 mg of free chlorine/liter in the different experiments, and at the end of the 20-min incubation, they were 0.22 mg/liter. After neutralization of the chlorine, the contents of the experimental and control tubes were diluted in infection medium for titration on Vero cells, which was carried out by the plate immunoperoxidase assay as previously described (15). In parallel, samples were tested by membrane enzyme immunoassay (MEIA) (see below) to determine the effect of chlorination on antigen detection.

MEIA. Cellulose nitrate filter membranes (0.45-μm pore size [Schleicher & Schuell] or 0.8-μm pore size [Pall Supor 800]) were used for filtration of control or tested water samples. Samples were mixed, and any coarse particles, if present, were removed by gravity drainage through several layers of viscose polypropylene material (this is a light gauze-like [but nonwoven] fabric composed of viscose and polypropylene fibers and used in clinics as disposable bed covers). Then a sample volume of 100 to 500 μl of reclaimed wastewater or 500 ml of drinking water was filtered (using a 96-place manifold system [S&S] with the 0.45-μm-pore-size membrane sheet or the 47-mm-diameter Pall Supor filter, respectively).

For each test, *S. negevensis*-infected Vero cell lysates or lysates of *A. polyphaga* persistently infected with *S. negevensis* (prepared as described below) served as controls. Twofold serial dilutions of these controls in distilled water (200-μl volumes) were filtered in duplicate through the manifold as calibration standards (Fig. 1B). Filters were processed as follows. They were frozen for 10 min at −70°C and incubated for 30 min at RT in 0.1% saponin in distilled water with vigorous shaking. The solution was removed, and the filter was frozen again at −70°C. The filter was then fixed for 15 min in a solution consisting of 30% methanol and 5% hydrogen peroxide, then fixed for 5 min in 70% ethanol, and fixed for 5 min in 96% ethanol. When the filter was almost dry, it was incubated for 45 min in a blocking solution containing 0.3% polyvinylpyrrolidone dissolved in TBS-Tween buffer, containing 10 mM Tris (pH 7.6), 150 mM NaCl, and 0.05% Tween-20, to which normal swine immunoglobulins (X-0906; DAKO), diluted 1:5,000, were added. The addition of swine immunoglobulins to the blocking buffer reduced the background due to nonspecific binding of rabbit or mouse immunoglobulins or of labeled secondary antibodies to biologic material on the filters.

For detection of the organisms trapped on the filters in the MEIA, the filters prepared as described above were incubated for 45 min at RT in rabbit anti-*S. negevensis* polyclonal hyperimmune serum diluted 1:20,000 in blocking solution. After washing three times for 5 min with TBS-Tween, the filters were incubated with affinity-purified horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (111-035-003; Jackson ImmunoResearch Laboratories, Inc.) diluted at 1:20,000 in blocking solution. After further washing, they were incubated in diaminobenzidine substrate solution. In each assay a duplicate control filter was prepared in parallel and processed as described above but omitting the serum incubation step. Only signals stronger than those obtained on the control filter were considered positive.

The same procedure was employed for detection of amoebic antigens with mouse polyclonal anti-*A. polyphaga* (at 1:5,000) and HRP-conjugated, affinity-purified anti-mouse antibodies (115-035-003; Jackson), diluted 1:2,000.

Preparation of purified *S. negevensis*, *Chlamydia trachomatis*, and *C. pneumoniae*. *S. negevensis*, *C. trachomatis* (L2/434/Bu), and *C. pneumoniae* (TW-183) were grown in Vero cells and purified by sucrose density gradient centrifugation as described previously (13, 17), except that the latter two organisms were grown in the absence of penicillin.

Preparation of positive controls for water studies. Vero cells infected with *S. negevensis* were scraped off the growth surface with glass beads at 3 to 5 days postinfection; aliquots were frozen at −80°C in the presence of 50% fetal calf serum. Infectivity titers were determined by titration on Vero cells in the plate immunoperoxidase assay as previously described (15). *A. polyphaga* organisms persistently infected with *S. negevensis* were grown in PYG medium containing antibiotics as detailed previously (12). *A. polyphaga* organisms were counted, and their *S. negevensis* infectivity was determined by titration on Vero cells, as described previously (12). The infected amoebae served as positive controls in MEIA in those assays in which the presence of both *S. negevensis* and amoebic antigens were determined.

Hyperimmune sera used in assays. Hyperimmune rabbit sera were prepared against *S. negevensis*, *C. trachomatis*, and *C. pneumoniae* as described previously for *C. trachomatis* and *C. pneumoniae* (7). Hyperimmune murine sera against *A. polyphaga* were produced by immunizing BALB/c mice with lysates of *A. polyphaga* grown in PYG.

List of bacterial strains and their American Type Culture Collection numbers. A panel of eight standard bacterial strains obtained from the American Type Culture Collection were used in developing the present protocol of the MEIA. They were as follows: *Branhamella* (*Moraxella*) *catarrhalis*, ATCC 25238; *Proteus vulgaris*, ATCC 33420; *Staphylococcus aureus*, ATCC 25923; *Escherichia coli*, ATCC 25922; *Staphylococcus epidermidis*, ATCC 12228; *Klebsiella pneumoniae*, ATCC 13883; *Haemophilus influenzae*, ATCC 19418; *Streptococcus pneumoniae*, ATCC 49619. A loop of each of the bacteria in the panel was suspended in water (concentration, about 10^7/ml) and stored frozen at −70°C.

PCR assays. DNA from drinking water samples was prepared as follows. A 500-ml volume of water sample was drained (by gravity) through a cellulose acetate filter (5-μm pore size, catalog no. 12342-47-N; Sartorius). The filter was incubated with Page's saline buffer (24) for 1 h. The solution containing the biological material was centrifuged for 20 min at 800 × *g* to sediment amoebae, and the precipitate was used to prepare DNA with the QIAamp kit (Qiagen). Assays for the detection of the *S. negevensis* genome by PCR were performed by using two sets of nested primers.

The first set consisted of primers ccF (CTT CGG GTT GTA AAG CAC TTT CGC) and ccR (CCC CGT CAA TTC TTT TGA GTT T), recognizing conserved chlamydial 16S ribosomal DNA (rDNA) sequences (433 to 457 and 933 to 945, respectively) and amplifying a 512-bp fragment, followed by nested ZpF and ZpR primers specific for *S. negevensis* Z (as previously described) (15), amplifying a 405-bp fragment.

A second set of nested primers consisted of AF and BR, amplifying a 1,099-bp fragment within the 23S rDNA of *S. negevensis* containing the large subunit intron (3), and IntD and IntR nested primers, amplifying a 338-bp fragment within this intron. If the DNA tested does not contain the intron, a 441-bp fragment is obtained with the AF and BR primers and no sequence is amplified by the IntF-IntR primer pair.

The use of the nested primers allowed increased sensitivity of the PCR assay when it was necessary; however, when DNA was abundant, primer set AF-BR, IntF-IntR, or ZpF-ZpR could also be used alone. PCR assays for the presence of amoebic sequences were carried out with the primers AmP1 and Amp2 described by Lai et al. (19) by using the cycling program described below for *S. negevensis*.

PCR amplifications were performed with *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan). Amplification conditions were 1 cycle for 6 min at 94°C; 30 cycles of 60 s at 94°C, 60 s at 53°C, and 60 s at 73°C; and then 1 cycle of 10 min at 73°C (3).

When amplification was with the ccF-ccR primers, the same amplification program was used, except that annealing was performed at 58°C instead of 53°C. When nested primers were used, the material amplified with the first pair of primers was diluted 1:100, of which 1 μl was used in the second reaction mixture. The high dilution was needed to ensure the specificity of the assay.

RESULTS

MEIA sensitivity and specificity. The sensitivity of the MEIA protocol was evaluated by determination of the number of *S. negevensis* inclusion-forming units (IFU) or number of amoebae present in the highest dilution of control material giving a positive signal. Using anti-*S. negevensis* serum diluted 1:20,000, 121 IFU of *S. negevensis* could be detected in the lysate and 468 infected amoebae could be detected. Uninfected *A. polyphaga* amoebae served as a negative control in the latter case. No signal was detected in the absence of anti-*S. negevensis* serum in the assay. When the same sample volumes were used for detection of amoebic antigen, the MEIA was able to detect 1,000 amoebae. *S. negevensis*-containing cell culture lysates and a filter used in the assay without the first antibody served as negative controls for the amoebic assay.

Figures 1 and 2 demonstrate the specificity of the MEIA for *Simkania* with respect to a number of common bacterial respiratory pathogens (Fig. 1) and members of the *Chlamydiaceae* (Fig. 2). A volume of 100 μl was used for the assay. None of the panel of standard bacteria gave a positive signal with antibodies to *S. negevensis* and affinity-purified goat anti-rabbit HRP conjugates (Fig. 1), nor did *C. trachomatis* or *C. pneumoniae* antigens under the same conditions (Fig. 2).

The possibility of demonstrating the presence of *S. negevensis* in water sources depends on the sensitivity and specificity of the detection assay; however, the significance of such detection depends on the continuous presence of the bacteria in the water source, which may be affected by various additional factors. Therefore, a number of experiments were performed to evaluate *S. negevensis* survival in the laboratory under simulated natural conditions.

Effect of chlorination on *S. negevensis* particles and on *S. negevensis* in persistently infected amoebae. In three separate experiments, the chlorinated suspension of *Simkania* particles had the same amount of infectivity as the control suspension that did not undergo chlorination. Control infectivity levels for the three experiments ranged from 1.2×10^4 to 2.8×10^5 IFU/ml. Survival values for *S. negevensis* particles ranged from 90 to 104% of the control untreated samples, with a mean of 96.8%. The variation obtained was within the experimental variation of titration. Similarly, *S. negevensis* in trophozoites of *A. polyphaga* were not sensitive to the treatment. In addition, chlorination treatment had no effect on antigen detection by MEIA (data not shown).

Survival in water of *S. negevensis* compared with *C. trachomatis*. *S. negevensis* particles, *S. negevensis*-infected amoebae, and *C. trachomatis* L2 particles were exposed for various periods of time to sterile distilled water at RT, and both infectivity and antigen detection were monitored (Fig. 3). *S. negevensis* particle infectivity was remarkably preserved even after 7 days of incubation (ranging from 8 to 15% in various experiments). *S. negevensis* titers in infected amoebae even increased significantly under the same conditions. The infectivity of *C. trachomatis* L2 particles was reduced to 65% within 1 h and was completely destroyed by 5 h of exposure to distilled water. However, antigens were detected by MEIA in all samples tested at all time points. Even when infectivity was no longer present, no significant change in the level of antigen detection was observed (data not shown).

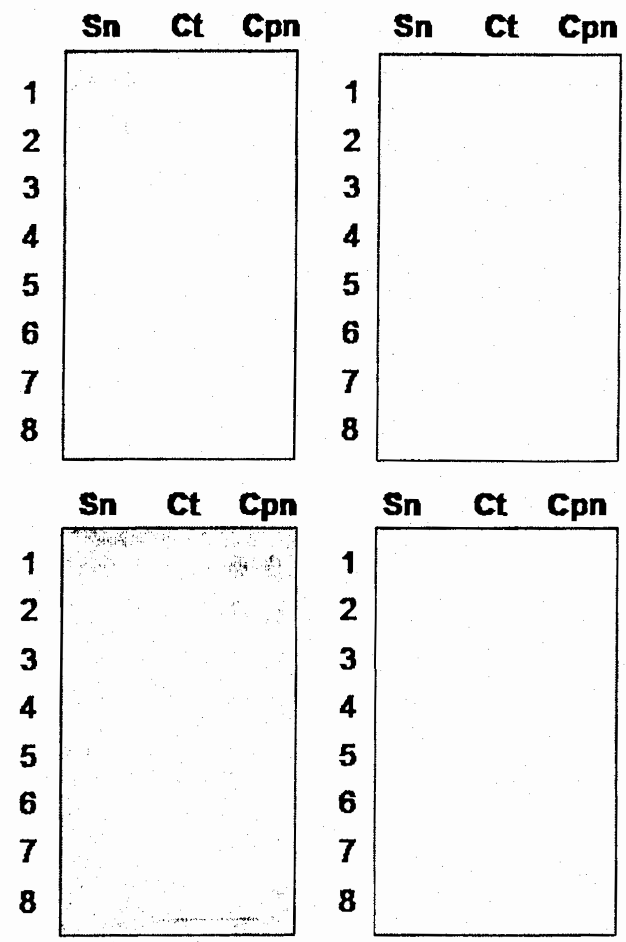

FIG. 2. Specificity of MEIA with respect to members of the *Chlamydiaceae*. Duplicate samples of 100-μl volumes of twofold dilutions of bacterial suspensions were applied to each of the membranes, as indicated. Sn, *S. negevensis*; Ct, *C. trachomatis* L2; Cpn, *C. pneumoniae*. Starting concentrations were as follows: *S. negevensis*, 4×10^4 organisms per dot; *C. trachomatis* L2, 2.5×10^4 organisms per dot; *C. pneumoniae*, 2.5×10^4 organisms per dot. Upper left panel, rabbit anti-*S. negevensis* serum used at 1:20,000; upper right panel, rabbit anti-*C. trachomatis* serum used at 1:20,000; lower left panel, rabbit anti-*C. pneumoniae* serum used at 1:20,000; lower right panel, serum diluent with secondary antibody only.

Detection of *S. negevensis* in water samples by MEIA. The MEIA was used to detect the possible existence of *S. negevensis* in drinking water or in reclaimed wastewater. On three differ-

41

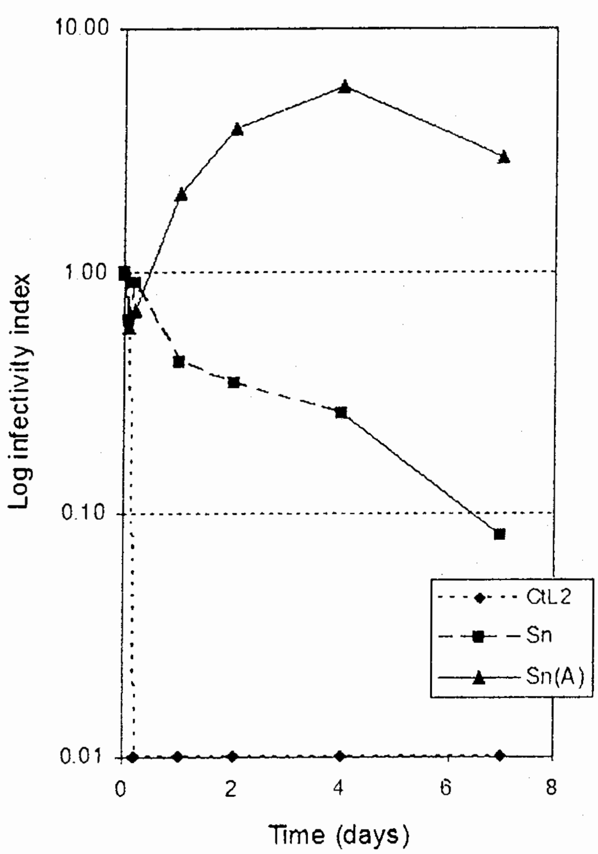

FIG. 3. Residual infectivity of *S. negevensis* and *C. trachomatis* after incubation for various times in sterile distilled water at RT. Sn, *S. negevensis*; CtL2, *C. trachomatis* L2; Sn(A), *S. negevensis* in persistently infected *A. castellani*. The infectivity index is the ratio of infectivity at a given time point to the infectivity at time zero.

ent occasions, at intervals of 1 month and then 2 months, drinking water samples were collected from houses located in various parts of Beer Sheva, with each location being visited on three different occasions. The results are shown in Table 1 (one sample was disqualified for inclusion in the table). As shown in Table 1, both *S. negevensis* and amoebic antigens were detected in most of the locations. In addition, 9 samples of drinking water from 3 satellite communities of Beer Sheva that obtain their water from aquifers were also examined on two different occasions, 10 days apart. In the majority of samples (7 of 9) both *S. negevensis* and amoebic antigens were detected by MEIA. In contrast, drinking water from Eilat, which is obtained by desalination (by reverse osmosis) had no traces of either *S. negevensis* or amoebic antigens (data not shown).

TABLE 1. Detection of *S. negevensis* and *Acanthamoeba* antigens in samples of drinking water[a]

Neighborhood no.	No. of positive samples/no. of samples tested for:	
	S. negevensis antigen	*Acanthamoeba* antigen
1	12/12	12/12
2	3/3	3/3
3	5/5	5/5
4	1/3	3/3
5	0/3	1/3

[a] Samples (500 ml) were collected from a number of houses located in Beer Sheva on three different occasions: 30 January 2003, 8 April 2003, and 6 May 2003) (days 1, 67, and 95, respectively).

Reclaimed wastewater is routinely examined by Israeli Ministry of Health laboratories before being approved for use in agriculture. Forty samples of such water obtained during a 1.5-month interval from various locations in the Negev were tested for the presence of *S. negevensis* and amoebic antigens by MEIA (Table 2). The majority of samples (39 of 40, or 97.5%) were shown to contain *S. negevensis* antigens. No samples were found to contain antigens of *S. negevensis* in the absence of amoebic antigens.

Detection of *S. negevensis* and *Acanthamoeba* DNA sequences in water samples by PCR. To confirm the finding of the *S. negevensis* antigen in drinking water, DNA was prepared from natural water samples by using the same volume of water as for the MEIA. The procedure for DNA preparation from water samples was optimized by using *A. polyphaga* persistently infected with *S. negevensis* and mixed with sterile water. All samples containing *Simkania* or *Simkania*-like antigens detected by the MEIA that were tested for the presence of *S. negevensis* DNA contained *Simkania* DNA as detected by PCR. The results from a representative experiment are shown in Fig. 4. Figure 4 also demonstrates the presence of amoebic DNA in both control and test samples (121-bp amplicon). Figure 5 shows the amplification of *S. negevensis* rDNA sequences with the ccF-ccR and ZpF-ZpR nested primers for a number of DNA samples obtained from water. It should be noted that the nested PCR proved to be more sensitive for the detection of *S. negevensis* DNA in water samples than the standard PCR previously described (2).

DISCUSSION

S. negevensis was initially isolated as a contaminant of cell cultures, and its natural host or hosts were unknown. In this study, evidence for the presence of *S. negevensis* or *Simkania*-like bacteria in drinking water or in reclaimed wastewater is presented for the first time. A MEIA was developed, and it was shown that this MEIA could be used for detection of *Simkania* antigens. Under the conditions used, there was no cross-reaction with members of the *Chlamydiaceae*. The method is simple and does not require sophisticated equipment or manipulations. PCR assays that require only one set of specific primers may not be sufficient to detect various unknown strains of the microorganism, more than one set may be needed, and in general, PCR assays are more expensive than the MEIA. Since the MEIA employs polyclonal antisera recognizing a wide range of *Simkania* antigens, it may be a more appropriate method for detection of various *Simkania* strains, the pathogenicity of which needs to be further investigated.

Chlamydia-like organisms other than *Simkania*, members of the family *Parachlamydiaceae*, have been shown to be present in the environment and to be able to grow and survive in protozoa, such as *Acanthamoeba castellani* and *Hartmanella* (9,

TABLE 2. Presence of *Simkania* and amoebic antigens (as detected by MEIA) in samples of reclaimed wastewater and quality characteristics of the water[a]

Treatment type/water quality[b]	No. of positive samples/no. of samples tested for:		BOD range	COD range	(T)SS range
	S. Simkania antigen	Amoebic antigen			
Intensive/good	7/7	6/6	9.5–26.1	28–62	5.5–26
Intensive/poor	2/2	2/2	104–107	170–181	33–144
Extensive/good	25/25	17/17	12.2–77.4	12.8–250	30–190
Extensive/poor	5/6	4/4	86.5–208.1	220–610	63–297

[a] BOD, biological oxygen demand; COD, chemical oxygen demand; (T)SS, total suspended solids.
[b] Intensive, activated sludge; extensive, oxidation ponds.

11), and lately, to replicate in human monocyte-derived macrophages (8).

Because we are able to grow *S. negevensis* in human and simian cells lines, we are able to isolate it from human clinical samples. We have also succeeded in isolating it from natural water samples (unpublished data). However, since the methods described in this study detect bacterial antigens or genomes only and are not capable of determining whether the organisms are viable, further studies are needed to understand the significance of the detection of *S. negevensis* or *S. negevensis*-like microorganisms in water. Aspects of such studies could include determination of the possible correlation between a high seroprevalence of antibodies to *S. negevensis* in healthy population samples and the presence of the organisms in the drinking water or agricultural irrigation water used in the same area. Sera obtained from a number of groups of healthy pregnant women (n = 192) living in the same areas from which water samples were obtained for this study showed an average prevalence of antibodies to *S. negevensis* of 75% (range, 50 to 92% for the different groups) (unpublished data), as assayed by enzyme-linked immunosorbent assay (6). The serum samples were from Jewish and Bedouin women living in rural and urban locations. The seropositivity ranges are consistent with published data (5, 6).

If the organisms found in drinking water are indeed viable, it is possible that their detection is of relevance to infection of the respiratory tract, in that drinking water is also generally used for oral hygiene, bathing, and showering, which can result in the formation of inhalable aerosols. Also, organisms found in drinking water may infect the oropharyngeal epithelium, thus gaining access to the respiratory and gastrointestinal tracts. In an ongoing study in our laboratory, *S. negevensis* strains isolated from children with respiratory disease are being compared to strains that may be isolated from their households.

Although laboratory experiments indicate that *S. negevensis* is more resilient than other similar bacteria, such as *Chlamydia*, we are well aware that under environmental conditions, bacteria and their antigens may have a shorter survival time due to the presence of microorganisms and factors such as various chemicals in the water. Clearly, it would be advantageous to have simple surrogate markers for viability of environmental chlamydia-like bacteria, but these are not presently available. The possibility of transmission of potential pathogens in drinking water is clear, but reclaimed wastewater may

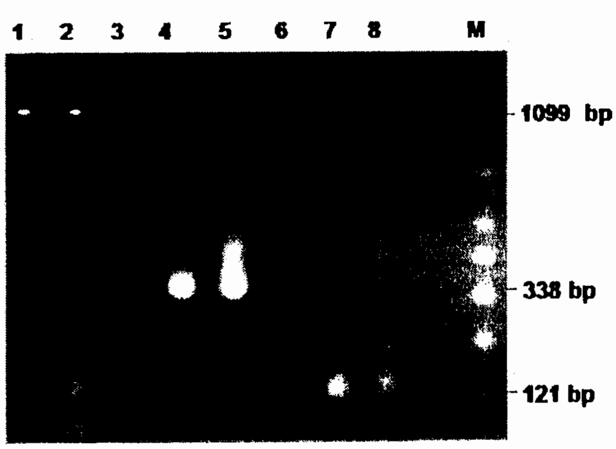

FIG. 4. Intron and amoebic amplicons detected by PCR assay of DNA prepared from water samples (lanes 2, 5, and 8) compared with DNA from purified cell culture-grown *S. negevensis* (lanes 1, 4, and 7), with the following primer pairs: AF-BR, lanes 1 and 2; intF-intR, lanes 4 and 5; amp1-amp2, lanes 7 and 8. M, marker (50- to 2,000-bp ladder; Bio-Rad).

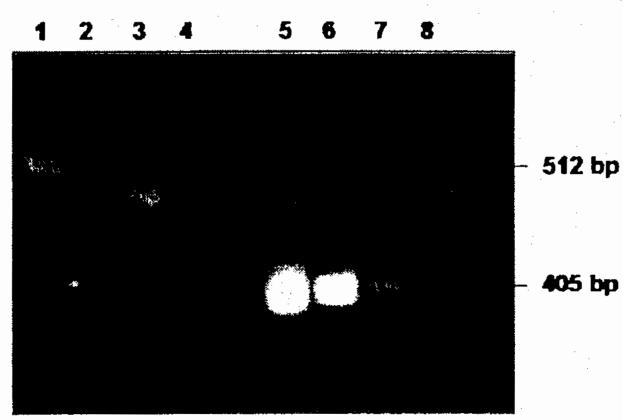

FIG. 5. Nested PCR for detection of 16S rDNA *S. negevensis* sequences in DNA prepared from water samples. The primers used were as follows: lanes 1 to 4, ccF-ccR; lanes 5 to 8, ZpF-ZpR (nested primers). Lanes 1 and 5: positive control; lanes 2 to 3 and 6 to 7: DNA prepared from two water samples; lanes 4 and 8, negative controls (no DNA).

be used for irrigation of crops and its pathogenic potential is also of concern. The significance of the ability of *S. negevensis* or *S. negevensis*-like microorganisms to survive in various water sources needs to be further studied to establish public health guidelines taking this into account. Future research will enable conclusions about the environmental, epidemiological, and health care implications of the very common existence of simkaniae in various water sources.

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44